

**STUDIES ON COMMON GENITAL HPV TYPES, HPV VARIANTS
AND HPV INTEGRATION IN THE DEVELOPMENT OF CERVICAL
CARCINOMA.**

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This thesis and all the experimental results presented herein, unless stated otherwise, are the product of my own work.

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SUMMARY

There has been considerable epidemiological and experimental evidence to support a role for human papillomavirus (HPV) in the development of cervical cancer, however the precise contribution of HPV remains controversial. Variation in prevalence of HPV types in cervical lesions has been found and there have been reports of a high prevalence of HPV in normal tissue. These variable findings have been attributed to several factors including the different detection and cervical sampling techniques used. In this study, a polymerase chain reaction (PCR) based assay for the detection of the common genital HPV types 6b, 11, 16, 18 and 33 was designed and optimised for both sensitivity and specificity. The assay was applied to 24 normal control cases, 20 cases each of cervical intraepithelial neoplasia (CIN) 1, CIN 2, CIN 3, 26 cases of squamous carcinoma, 16 cases of adenocarcinoma and 4 cases of mixed adenosquamous carcinoma. HPV DNA was found in 25% of CIN 1 cases, 60% of both CIN 2 and CIN 3 cases and in 80% of cervical carcinomas. A novel finding was that no HPV DNA was detected in the normal control cases. No HPV 6b or 33 DNA was found and HPV 11 was detected in only 5% of CIN 1 cases. HPV 16 and HPV 18 were seen with increasing frequency through the spectrum of cervical disease. The results of these assays support a role for HPV 16 and 18 in neoplastic progression and emphasise the biological similarity of CIN 2 and CIN 3 and their divergence from CIN 1.

The prevalence of HPV 16 and HPV 18 in cervical neoplasms was compared. HPV 16 was found in more squamous carcinomas (58%) than HPV 18 (23%), whereas in adenocarcinomas - which have a poorer prognosis than squamous carcinomas - both HPV types showed an equal prevalence (44%). Thus, in terms of cancer cell differentiation HPV 16

appeared to confer a better prognosis than HPV 18. The frequency of HPV 16 in all cancers and CINs gave a CANCER/CIN prevalence ratio of 1.4 (54%/38%) as compared to one of 3.75 (30%/8%) for HPV 18 - a 2.6 fold difference - indicating either a greater risk of, or more rapid progression to malignancy associated with HPV 18. These findings suggest that HPV 18 is more aggressive than HPV 16.

While HPV types 16 and 18 appear to contribute to the neoplastic process not all cases of HPV infection will progress to invasive carcinoma indicating the likely involvement of additional events or factors. It has been suggested that HPV variants with sequence alterations to the upstream regulatory region (URR) may contribute to the neoplastic process through an altered pattern of gene expression. Little is known of their prevalence in cervical lesions. In this study a PCR assay was designed specifically to detect large scale sequence alterations in the HPV 16 and 18 URR. The assay was applied to 24 cases of CIN (20 HPV 16, 4 HPV 18) and 34 cases of carcinoma (22 HPV 16, 11 HPV 18, 1 HPV 16 and HPV 18). No URR alterations were found indicating that HPV variants are not widespread in cervical lesions.

HPV DNA of types 16 and 18 has been found integrated in a monoclonal pattern into the host genome in a high proportion of cervical carcinomas and cell lines derived from them. It is thought that this event may disrupt the replicative pattern of viral gene expression and effect sustained or increased transcription of the transforming E6 and E7 genes, contributing to the neoplastic process. However it is unclear at which stage in cervical carcinogenesis this event occurs. Here, a PCR based assay was designed to determine the physical state of HPV 16 DNA. This

assay differs from previously described methods in that it was designed to be applicable to small clinical samples such as punch biopsies of CIN. The assay has been successfully applied to cloned HPV plasmid DNA and is now ready to be used to determine the proportion of CIN 2, CIN 3 and microinvasive carcinomas that contain integrated HPV 16.

TABLE OF CONTENTS

DECLARATION	I
ACKNOWLEDGEMENTS	II
SUMMARY	III
CHAPTER 1: INTRODUCTION	1
1.1 Cervical cancer and precursor lesions	2
1.2 Aetiology of CIN and cervical cancer	5
1.3 The Biology of HPV	7
1.3.1 Virion properties and Classification	7
1.3.2 Papillomavirus infection	8
1.3.3 Genome Organization and Function	8
1.4 Papillomavirus Transformation	17
1.5 Mechanism of HPV Transforming Activity	20
1.6 Regulation of HPV Gene Expression	21
1.6.1 Organisation of the URR	21
1.6.2 Regulation by E2	22
1.6.3 Regulation by Cellular Factors	25
1.7 Alterations in the Viral Upstream Regulatory Region (URR)	27
1.8 Physical State of HPV DNA in Cervical Tumours	29
1.8.1 Effects of integration on the viral genome	30

1.8.2 Effects of integration on the cell genome	31
1.9 HPV and Cellular Oncogenes	31
1.10 Additional Risk Factors for Cervical Neoplasia	32
1.11 A Multistage Model for Cervical Carcinogenesis	33
1.12 Aims	37
CHAPTER 2: MATERIALS AND METHODS	38
2.1 Clinical Material	39
2.2 Histopathological Examination	39
2.3 Oligonucleotide Selection and Synthesis	40
2.4 Preparation of Materials for PCR	40
2.5 Polymerase Chain Reaction	42
2.6 Procedures to Minimise the Risk of Contamination	42
2.7 Amplification of Reference Gene	43
2.8 Dot Blot Hybridisation	43
2.9 Restriction Enzyme Mapping	45
2.10 Anchor PCR	45
CHAPTER 3: DESIGN AND OPTIMIZATION OF A PCR DETECTION ASSAY	47
3.1 Introduction	48
3.2 Results	50
3.2.1 Design of oligonucleotides for the detection assay	50
3.2.2 Optimization of condition for the detection assay	56

3.2.3 Validation of the detection assay for HPV type specificity	58
3.2.4 Validation of the detection assay for sensitivity	60
3.3 Discussion	65
CHAPTER 4: DETECTION OF HPV IN CERVICAL NEOPLASIA	69
4.1 Introduction	70
4.2 Cases studied	70
4.3 Results	71
4.4 Discussion	76
CHAPTER 5: DETECTION OF LARGE SCALE SEQUENCE ALTERATIONS IN THE HPV URR	90
5.1 Introduction	91
5.2 Assay design	94
5.3 Results	98
5.4 Discussion	105
CHAPTER 6: DESIGN OF A PCR-BASED ASSAY TO DETERMINE THE PHYSICAL STATE OF HPV DNA IN CERVICAL LESIONS	109
6.1 Introduction	110
6.2 Assay design	112
6.2.1 PCR analysis	112
6.2.2 Anchor-PCR analysis	114

6.2.3 Primer Design	118
6.2.4 Optimization of conditions for initial PCR	118
6.2.5 Selection of restriction enzyme sites	121
6.3 Results	121
6.4 Discussion	123
CHAPTER 7: DISCUSSION	130
BIBLIOGRAPHY	141

CHAPTER 1
INTRODUCTION

Cervical cancer is the largest single cause of cancer related deaths among women in developing countries and worldwide is second only to breast cancer with an estimated 459,000 new cases worldwide each year (Stanley et al., 1987). Cervical neoplasia is a multistage disease which has a precursor of malignancy recognised morphologically as cervical intraepithelial neoplasia (CIN). In countries such as Denmark, Finland and parts of Scotland, systematic cytological screening programs aimed at detecting and treating CIN have been effective in reducing the incidence of mortality from cervical cancer (Report of the Intercollegiate working party on cervical screening, 1987). However, UK statistics show an increase in the incidence of both cervical cancer and CIN, particularly in young women (Scottish cytology statistics, 1986; Report of the Intercollegiate working party on cervical screening, 1987; Cancer registration statistics, Scotland, 1990).

1.1 Cervical Cancer and precursor lesions

Cervical cancer is heterogeneous in its patterns of differentiation and can be divided histopathologically into squamous carcinoma, adenocarcinoma and adenosquamous carcinoma. Squamous carcinomas are defined as tumours composed of cells resembling those of the squamous epithelium whereas, adenocarcinomas show glandular differentiation and are often recognised by their capacity to secrete mucin. Adenosquamous carcinomas may represent the centre ground of a spectrum of bipotentiality as they contain both squamous and adenocarcinomatous elements and behave biologically in a very similar manner to squamous carcinoma with mucin production (Buckley and Fox, 1989). Squamous carcinomas have been more extensively studied than adenocarcinomas and adenosquamous carcinomas probably owing to the fact that this type

constitutes the majority of all cervical cancers.

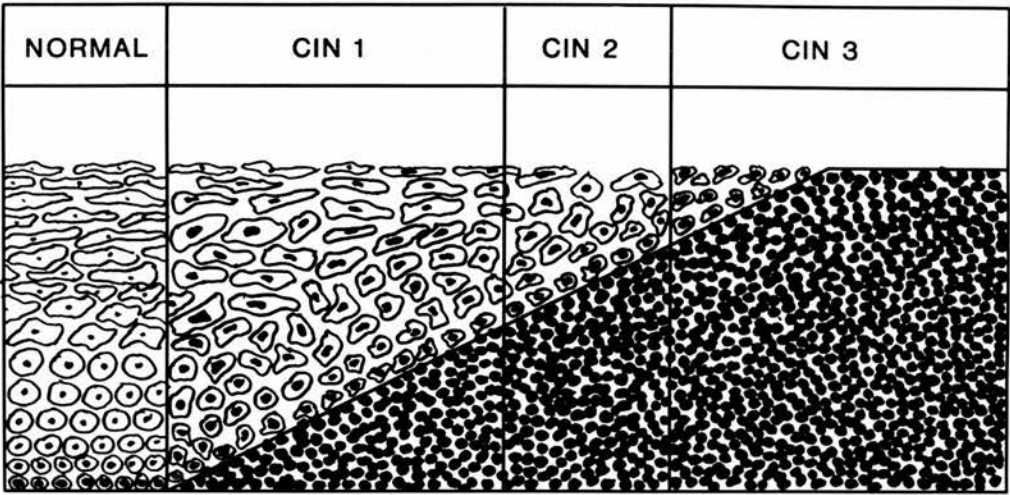
Squamous carcinoma has a precursor stage known as CIN which represents a spectrum of disorder in the epithelium. CIN can be categorised into arbitrary grades one, two or three on the basis of whether disordered cells comprise one third, two thirds or the entire thickness of the epithelium respectively (figure 1.1). Invasive cancer develops from CIN when disordered cells breach the underlying basement membrane invading the stroma.

Evidence supporting this sequence of events has come from epidemiological studies which have found identical risk factors for CIN and invasive lesions (Rotkin, 1973; Vessey et al., 1983; Greenberg et al., 1985) and prevalence studies which have found the age distribution of the different lesions to be consistent with multistage progression in that the more severe grades of CIN tend to be found in older women. A retrospective study of untreated patients with precancerous lesions of the cervix found that in 27% of cases, invasive cancer developed within ten years (Peterson, 1956). A similar study found that 19% of women with CIN lesions may develop invasive cancer if untreated for two years or more (Kinlen and Spriggs, 1978). More recently Nasiell et al (1983) found that 30% of 894 women with CIN 2 lesions developed carcinoma in situ (CIN 3) within 50 to 78 months.

Despite the existence of a spectrum of change, development of invasive carcinoma may not necessarily involve sequential progression through a stable CIN 1, 2 and 3 stage. Possible pathways may involve progression to invasive cancer from normal epithelium via a stable CIN 2 or CIN 3

Figure 1.1

Diagrammatic representation of the spectrum of change in the cervical epithelium recognised morphologically as cervical intraepithelial neoplasia (CIN). Increasing severity of CIN is characterised by a progressive increase in the number of disordered cells until the full thickness of the epithelium is involved. Data modified from Ferenczy and Winkler (1987).



stage, or from normal epithelium via a CIN 1 stage only or even directly from normal epithelium (Reid and Fu, 1986). Furthermore, there are many cases of CIN which do not progress in the lifetime of the woman, some persist and others regress. While the likelihood for regression decreases with increasing severity of CIN there is no way of predicting the clinical outcome of any individual lesion.

Adenocarcinoma in situ (AIS) and glandular atypia have been proposed as precursor lesions of glandular carcinoma on the basis of their morphological features - which are similar to adenocarcinoma without stromal invasion - and also the age distribution of the lesions which is in keeping with multistage progression (Wells and Brown, 1986). However, there has been no direct proof that glandular atypia will progress to AIS and then finally to invasive cancer. In addition, it is possible that adenocarcinoma may develop from CIN and that AIS may progress to squamous carcinoma.

1.2 Aetiology of CIN and Cervical Cancer

The epidemiology of both CIN and cervical cancer show a clear correlation between the disease and sexual behaviour. Several risk factors have been identified including multiple sexual partners and early age at first intercourse (Brinton and Fraumeni, 1986; Parazzini et al., 1988), suggesting an aetiological role for a sexually transmitted infectious agent. While a number of candidates have been implicated, recent years have seen the accumulation of considerable evidence supporting a key role for the genital human papillomaviruses (HPV) in the neoplastic process.

Some of this evidence has been provided by epidemiological and HPV

prevalence studies which have found HPV DNA in CIN lesions in punch biopsies and cervical smears and in up to 90% of squamous cervical cancers (Arends et al., 1990). HPV DNA has also been found by some investigators in glandular cervical carcinomas and tumours of mixed differentiation (Tase et al., 1988).

It might be argued that HPV is present in these lesions as a passenger and that the virus merely has a predilection for infecting CIN. However, evidence to the contrary comes from the fact that different HPV types have been found in association with specific lesions. HPV 6 and HPV 11 are found mainly in low grade CIN (CIN1) and warts whereas HPV 16 and HPV 18 are found in higher grade CIN (2-3) and invasive carcinomas (Gissmann, 1984; Brescia et al., 1986; Pater et al., 1986; Syrjanen et al., 1987). A comparison of the spectrum of condyloma, CIN, carcinoma-in-situ (CIS) and invasive carcinoma has shown a progressive increase in the prevalence of HPV 16 and 18 and a corresponding decrease in HPV 6 and 11 (Gissmann, 1984). On this basis, HPV types 6 and 11 have been categorised as low risk and HPV types 16 and 18 as high risk. Furthermore, HPV 18 appears to be associated with glandular cancers of the cervix and HPV 16 with squamous carcinoma (Wilczynski et al., 1988; Okagaki et al., 1989; Leminen et al., 1991). HPV 18 may also be associated with a more aggressive form of cervical cancer than HPV16 based on the relatively low prevalence of HPV 18 in CIN compared with cervical cancers (Kurman et al., 1988).

There are over sixty HPV types now identified and several more have been detected in cervical lesions, albeit less frequently than types 6, 11, 16 and 18. These include HPV types 30, 40, 42, 43, 44 and 58 which are

associated with CIN and types 31, 33, 35, 39, 45, 51 and 52 which are associated with both CIN and cervical carcinoma (de Villiers, 1989). However, there are still some cervical cancers which are negative for HPV DNA. While these cancers may be genuinely unrelated to HPV infection it is also possible that they contain HPV DNA at a copy number that is too low for detection by previously used methods. It is also possible that these apparently HPV negative carcinomas may contain HPV types which are yet to be characterized.

1.3 The Biology of HPV

Whilst epidemiological evidence strongly suggests a role for HPV in the development of cervical carcinoma the precise nature of the involvement is unclear. The study of HPV biology has contributed to the understanding of the possible mechanisms by which viral infection may influence cellular genes and gene products.

1.3.1 Virion Properties and Classification.

The papillomaviruses are members of the Papovaviridae. They are unenveloped and have cubic capsids which contain closed circular double stranded DNA of approximately 8kb in length (Pfister, 1987). They are classified in accordance with the host species they infect and by the degree of cross hybridisation in the liquid phase. Different PV types have less than 50% cross hybridisation (Coggin and zur Hausen, 1979), whereas different subtypes have greater than 50% cross hybridisation but with different restriction enzyme cleavage patterns. The most extensively studied of the PVs are the bovine papillomaviruses (BPV) and the human papillomaviruses (HPV). The different types of HPV can be grouped according to the sites of their associated lesions (de Villiers, 1989). The

genital PVs are considered a family as a single family. Other families include those infecting the skin and those causing fibropapillomas.

1.3.2 Papillomavirus Infection

The PVs infect the squamous epithelium of skin and mucosae producing proliferations with different growth patterns dependent upon the site of infection and virus type. Initial infection requires direct physical access to basal cells such as occurs at local abrasions or at the transformation zone of the cervical squamocolumnar junction. Subsequent viral replication appears to be very closely linked and possibly dependent on epithelial cell differentiation (Bedell et al., 1991). In the lower layers of the squamous cervical epithelium the early genes which regulate DNA synthesis are expressed (Pfister, 1987) whereas the expression of capsid proteins and packaging of viral genomes are limited to the most highly differentiated upper layers indicating that different stages of the viral life cycle may require specific factors only provided by sequential differentiated stages of epithelial tissue. Until recently study of the mechanisms by which epithelial differentiation could influence papillomavirus replication was hindered due to inadequate culture methods. The recently developed *in vitro* raft culture systems and xenografting techniques may help to overcome this problem by mimicking the differentiation-specific processes seen *in vivo* thus allowing the study of many aspects of the viral life cycle including actual virus production (Merrick et al., 1992).

1.3.3 Genome Organization and Function

The PVs infecting animals and humans all exhibit a very similar organization of the genome with at least ten potential open reading frames (ORFs) all occurring in similar positions on the same DNA strand (Chen et

al., 1982; Schwarz et al., 1983; Seedorf et al., 1985; Matsukura et al., 1986) (figure 1.2). The genome can be functionally divided into three regions: a non coding region commonly termed the upstream regulatory region (URR) which contains transcriptional control sequences and the origin of replication, followed by two coding regions; an early coding (E) region containing genes E1 - E8 and a late coding region containing genes L1 and L2. The transcription of viral DNA into messenger RNA (mRNA) includes the formation of both unspliced and spliced mRNA and more than one protein may derive from the same coding sequence (Pettersson et al., 1987). Functions have now been assigned to almost all of the ORFs. This has been achieved primarily by experiments in which the ORF of interest is deleted, mutated or subcloned and overexpressed (from a surrogate promoter in the absence of the other ORFs), using either human or bovine papillomavirus genomes. The assigned functions of the HPV ORFs are summarised in table 1.3.

The L1 and L2 open reading frames

This region contains two large ORFs, L1 and L2, which code for viral structural proteins. L1, the most highly conserved viral gene codes for a major capsid protein. The L2 ORF codes for a similarly sized but poorly conserved minor capsid protein (Pilacinski et al., 1984; Rose et al., 1990).

The E1 open reading frame

The BPV 1 E1 ORF encodes sequences for two complementation groups involved in different aspects of viral replication. The 3' R gene portion is required for early replication events possibly encoding a positive replication factor required for initial amplification of DNA soon after infection. Mutation of this region always leads to integration of the virus

Figure 1.2

Genome organization of BPV 1 (Chen et al, 1982), HPV 6 (Schwarz et al, 1983) and HPV 16 (Seedorf et al, 1985; Matsukura et al, 1986). The open bars represent open reading frames (ORFs) for each of the three potential translation frames. E and L denote early and late region ORFs respectively. Solid bars indicate the upstream regulatory regions. There is a translation start codon (ATG) at or near the beginning of almost all the coding sequences. All ORFs are capable of coding for a polypeptide uninterrupted by a stop codon.

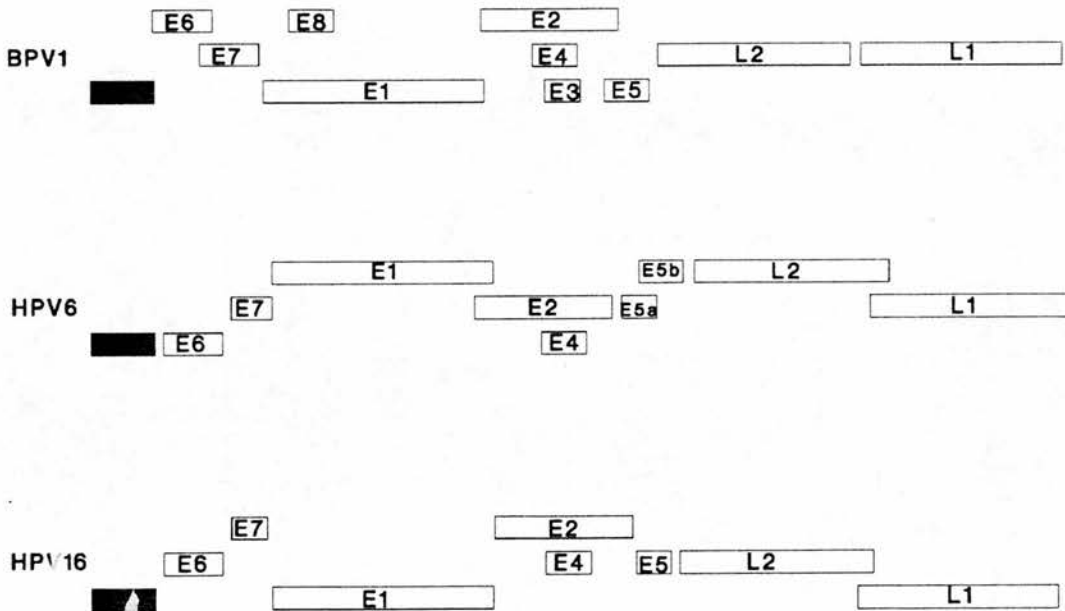


Table 1.3

Table of HPV ORF function (see text for details).

ORF	Properties
E1	virus replication, episomal maintenance
E2	positive and negative transcriptional control
E4	late function; virus release
E5	membrane protein; transforming function?
E6	transforming gene
E7	transforming gene
L1	major capsid protein
L2	minor capsid protein

into the host genome. The 5' M gene portion encodes a *trans*-acting factor which represses BPV 1 replication (Berg et al., 1986). Interestingly, two HPV 11 cDNAs with the potential to encode two fusion proteins incorporating the putative E1M domain have been recovered using retroviral gene transfer (Rotenberg et al., 1989; Chiang et al., 1991). Functional analysis suggested that these proteins are both transcriptional repressors (Chiang et al., 1991).

The E2 open reading frame

E2 is thought to play a key role in the negative and positive control of viral transcription. Using the BPV 1 model the E2 ORF has been found to encode at least 3 transcriptional regulatory proteins which all share the same carboxyl terminal but have different functions: a full length E2 gene product (E2) transactivates the BPV 1 E6-E7 promoter (Spalholz et al., 1985) a short C-terminal E2 gene product (E2-C) represses transactivation by the full length E2 gene product (Lambert et al., 1987a, 1987b; Cripe et al., 1987); a recently defined E2/E8 fusion protein, is also a transcriptional repressor (Lambert et al., 1987a, 1989). Proteins corresponding to these gene products have been identified in BPV 1 transformed cells (Hubbert et al., 1988). The C-terminal region of both the HPV 16 and HPV 11 E2 ORFs have the coding capacity for a repressor function similar to BPV 1 E2-C, however the actual protein has not been identified in infected cells (Cripe et al., 1987; Chin et al., 1988).

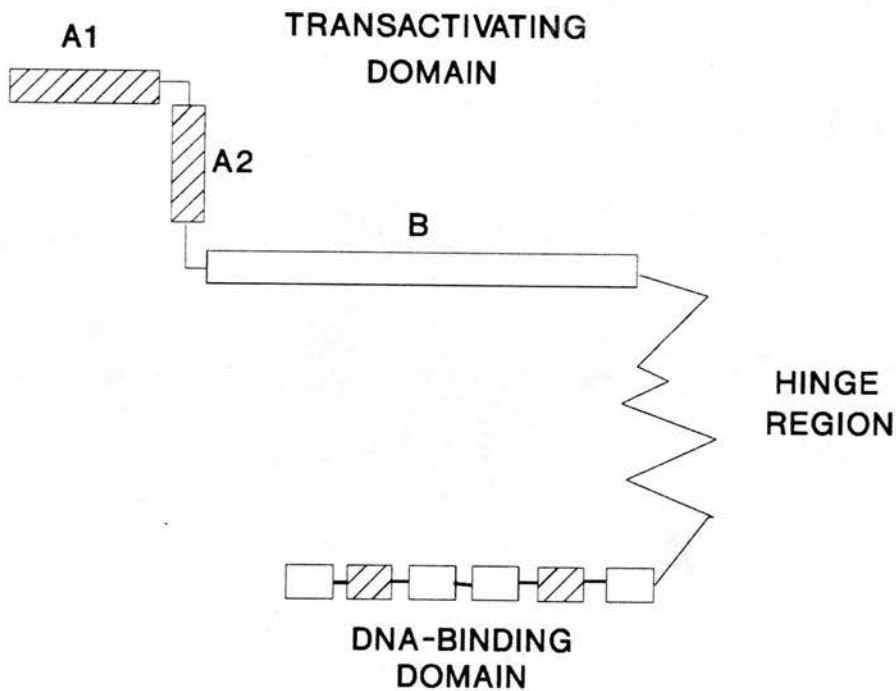
Transcriptional regulation by the E2 proteins is mediated by direct binding to the palindromic sequence ACCN₆GGT (E2 binding sites or E2BS) (Haugen et al., 1987; Spalholz et al., 1987; Androphy et al.,

1987a; Moskaluk and Bastia, 1988a; McBride et al., 1988) found in all the known papillomavirus URRs (Dartmann et al., 1986), with the exception of HPV41, which contains a modification of this sequence (Hirt et al., 1990). The number and location of the E2 binding sites differs among virus types. Although there is little homology between PV E2 proteins, analysis of the alignment of the amino acid sequences of 10 E2 proteins reveals three distinct regions: two partially conserved domains at the N and C termini of the protein and a hinge region variable in size and sequence in the middle (Giri and Yaniv, 1988) (figure 1.4). Mutational analysis has allowed particular functions to be ascribed to the three domains (Giri and Yaniv, 1988; McBride et al, 1989). The C terminal domain is responsible for DNA binding and dimerization (Giri and Yaniv, 1988). C terminal E2 polypeptides synthesized *in vitro* have been shown to bind the perfect palindromic sequence ACCGN₄CGGT and with lower affinity the degenerate palindromic sequence ACCN₆GGT (Androphy et al, 1987a; McBride et al, 1988; Hawley-Nelson et al, 1988; Moskaluk and Bastia, 1988a; 1988b). Interestingly, the C terminal domain contains motifs capable of forming "leucine zipper" type structures (O'Shea et al., 1989). Such structures are involved in dimerization of certain known DNA binding proteins (Landshultz et al., 1988), however their involvement in E2 dimerization and DNA binding is yet to be established.

It seems likely that the E2/E8 and E2-C repressors which share a common C terminal will also share dimerization and DNA binding functions. Thus these repressor proteins could exert their effect by competing with E2 for binding sites or also by the formation of heterodimers between repressor and full length E2 protein (Lambert et

Figure 1.4

Computer predicted secondary structure of the full length E2 protein. Hatched bars represent conserved alpha helices. Two long helices in the transactivating domain are named A1 and A2. Open bars represent conserved beta strands. The long beta strand in the transactivating domain is labelled B. Solid lines indicate sequences of undetermined structure. The zig-zag line indicates the absence of reliable structural information for the "hinge" region. Data redrawn from Giri and Yaniv (1988).



al., 1987b; Haugen et al., 1988; McBride et al., 1989).

The N terminal consists of 2 negative amphipathic alpha helices and a small beta strand. Mutational analysis showed the alpha helices to be essential for transactivation while the beta strand had a less important effect (Giri and Yaniv, 1988). Negative amphipathic helices are thought to be directly involved in transcription through protein-protein interactions with transcription factors and are a common feature among transactivator proteins (Giniger and Ptashne, 1987). Although binding affinity of E2 proteins is similar with one or two tandem repeated E2BSs a minimum of two is required for a functional E2 responsive enhancer (Gius et al., 1988; Hawley-Nelson et al., 1988; Spalholz et al., 1988). A recent report has shown that BPV 1 E2 binds to the E2BS as a dimer and then multimerizes further into a stable protein-DNA complex forming DNA loops if the palindromes are far apart (Knight et al., 1991). This looping out could bring distant E2 proteins close to the promoter enabling them to interact with bound cellular transcription factors.

Although the precise mechanism by which E2 activates transcription is unknown it is possible that E2 may stabilize the transcription initiation complex by protein - protein interactions between bound E2 and transcription machinery such as TFIID and RNA polymerase II.

The E4 open reading frame

The E4 ORF lies within the E2 ORF but in a different reading frame. E4 has been found to be dispensable for many of the early functions and a late function has been suggested for the gene (Hermonat and Howley,

1987; Neary et al., 1987). The E4 protein of HPV 1a has been isolated as a cytoplasmic protein and has been found to represent up to 30% of total cellular protein in HPV 1a induced warts (Doorbar et al., 1986). Another study has shown that the HPV 16 E4 ORF is expressed as a cytoplasmic protein in detectable quantities only where episomal replication is accompanied by late gene expression (Crum et al., 1990). The *in vitro* expression of an HPV 16 E1⁺E4 fusion protein in epithelial cells has been shown to disrupt cytokeratin organization providing support for a recent suggestion that E4 may play a role in altering the normal cytokeratin matrix of an infected cell thus facilitating the release of virus particles when the cell is shed (Doorbar et al., 1991).

The E5, E6 and E7 open reading frames

The role of the HPV E5 gene in the virus life cycle is not well understood. Sequence analysis of the HPV 16 genome has revealed that E5 encodes a small, strongly hydrophobic protein containing 83 amino acids (Bubb et al., 1988). Recent studies have shown that the HPV 16 E5 gene can interact with the cellular epidermal growth factor receptor to induce transformation of NIH3T3 cells (Pim et al., 1992). Furthermore, studies by Leechanachi et al (1992) have suggested that the E5 gene from HPV 16 is an oncogene which transforms cells in part through enhancing signal transduction from growth factors to the nucleus.

The HPV E6 and E7 genes encode the major transforming proteins (Yutsudo et al., 1988). Their function and mechanism of transforming activity are discussed later.

1.4 Papillomavirus Transformation

In the normal stratified squamous epithelium of the cervix, cells stop dividing as they leave the basal layer and undergo an ordered pattern of differentiation. The development of CIN in these tissues involves a disruption of this pattern. One manifestation is an alteration in nuclear and cytoplasmic maturation initially more obvious in the lower layers of the epithelium in low grade CIN but, spreading to involve the entire thickness of the epithelium in high grade CIN. The abnormal cells are characterized by architectural disorientation, enlarged and irregularly shaped nuclei and increased and abnormal mitoses. The potential of the papillomaviruses to alter cellular morphology and growth properties in such a way has been widely investigated *in vitro* using transformation assays and more recently, organotypic raft culture (Merrick et al., 1992).

The high risk HPV types (16 and 18) and low risk HPV types (6 and 11) differ in their ability to transform both rodent and human cells. HPV types 16 and 18, but not types 6 and 11, can transform rodent immortalized cell lines C127 and NIH3T3 to tumorigenicity (Watts et al., 1984; Yasumoto et al., 1986; Bedell et al., 1987). In addition, high risk HPV types 16, 18, 31 and 33 are capable of cooperating with activated *ras* oncogene to transform primary rodent kidney epithelial cells - a feature very rarely seen in the low risk types (Crook et al., 1988; Storey et al., 1988, 1990b).

However the natural target cell of HPV is not the rodent fibroblast or epithelial cell but the human keratinocyte. The introduction of HPV DNA of types 16, 18, 31 and 33 can immortalize human genital keratinocytes although the resultant cell lines are not tumorigenic (Durst et al., 1987a; Pirisi et al., 1988; Woodworth et al., 1988, 1989). When such HPV16- and 18- transfected keratinocytes are cultured on collagen rafts *in vitro* they fail to differentiate normally and show histological abnormalities similar to genital intraepithelial neoplasia *in vivo* (McCance et al., 1988; Blanton et al., 1991). Full transformation of HPV-16 immortalized cell lines requires the introduction of an activated viral Harvey ras gene and generates cells capable of forming cystic human squamous cell carcinomas (DiPaolo et al., 1989).

While a low level of transforming activity has been mapped to the E2-E4-E5 region of both HPV 16 and HPV 18 (Vousden and Jat., 1989; Bedell et al., 1989), it is the E6 and E7 genes which are primarily involved in the transformation of cultured cells. In high risk HPV types the E6-E7 subgenomic fragment alone was found to be sufficient to immortalize rodent cell lines (Bedell et al., 1987; Matlashewski et al., 1987; Vousden et al., 1988). Furthermore, when E6-E7 immortalized cells were cultured in a raft system morphological changes occurred similar to those described above (Hudson et al., 1990). Studies on the independent immortalizing activity of the HPV 16 E6 and E7 genes in primary rodent cells found E6 less efficient than E7 (Kanda et al., 1988; Vousden and Jat, 1989). The E7 sequence of the high risk HPV types can co-operate with mutated c-Ha-ras to transform primary rodent cells (Storey et al., 1988; Crook et al., 1988; Chesters et al., 1990). Although the E7 region of HPV types 16 and 18 is sufficient for the immortalization of primary

human keratinocytes E6 contributes significantly to the efficiency of the E7 immortalizing function (Hudson et al., 1990; Halbert et al., 1991).

Further evidence of the importance of E6 and E7 is derived from the observation that the high risk HPV types 16 and 18 differ in their capacity to immortalize human keratinocytes, with HPV 18 10-50 fold more active than HPV 16 (Villa and Schlegel, 1990). This differential activity has been mapped to the viral URR which controls expression of the E6 and E7 transforming genes (Romanczuk et al., 1991).

In support of the view that the transforming potential of the HPVs lies primarily within the E6 and E7 genes, are reports that both human cervical carcinomas and carcinoma-derived cell lines contain HPV E6 and E7 mRNA and proteins (Smotkin and Wettstein, 1987; Baker et al., 1987; Seedorf et al., 1987; Androphy et al., 1987b; Shirasawa et al., 1987; Pater and Pater, 1988).

The HPV E6 and E7 proteins all contain the amino acid motif Cys- X-X- Cys which mediates zinc binding and is implicated in DNA binding (Cole and Danos, 1987; Barbosa et al., 1989; Grossman and Laimins, 1989). This motif repeated at regular intervals in BPV 1 E6 protein is essential for BPV E6 transforming activity (Vousden et al., 1989). Recent point mutational analyses of the HPV 16 E7 protein have demonstrated that Cys-X-X-Cys motifs contribute to the transforming potential of E7, but are not essential to it (Edmonds and Vousden, 1989; Storey et al., 1990).

Interestingly, DNA sequence analysis predicts the existence of E6*

mRNAs generated by internal splicing events in the high risk genital HPV types (Schneider-Gadicke and Schwarz, 1986). However, such proteins have not yet been identified and no function has been assigned.

1.5 Mechanism of HPV transforming activity

Recent biochemical experiments have shown a mechanism by which the viral E6 and E7 proteins may affect cellular proliferation. The E7 protein of oncogenic HPV types shares an amino acid motif with the SV40 large T antigen, adenovirus E1A protein and v- and c-*myc* oncoproteins. This motif has been shown to encode the transforming function of SV40 large T antigen and bind to the protein encoded by retinoblastoma (Rb) oncosuppressor gene (Figge et al., 1988; Figge and Smith, 1988; Dyson et al., 1989; Munger et al., 1989). It has been suggested that viral transforming proteins such as E7 may induce transformation by binding to the Rb protein and neutralising its growth inhibitory effects thereby allowing uncontrolled proliferation. In support of the importance of Rb inactivation in tumours, the Rb gene has been found to be mutated in a variety of human cancers including sarcomas and breast cancers (Horowitz et al., 1990).

Similarly, the E6 protein of HPV types 16 and 18 binds to the product of a second tumor suppressor gene p53 *in vitro* (Werness et al., 1990) as does SV40 large T antigen and adenovirus 5 E1B protein (Sarnow et al., 1982). However unlike these other oncoproteins, E6 has been shown to promote the degradation of p53 (Scheffner et al., 1990). This occurs through the ubiquitin-dependent proteolysis system and is mediated by a cellular monomeric protein of approximately 100kDa (Huibregtse et al., 1991). This discovery would account for the unexpectedly low levels of

p53 protein found in some of the cervical carcinoma cell lines and HPV-immortalized squamous epithelial cell lines (Matlashewski et al., 1986).

Thus, HPV 16 and 18 show a striking parallel with SV40 and adenovirus. All have proteins that bind and inactivate p53 and Rb oncosuppressor proteins. This conserved dual effect of three DNA tumour viruses may play an important role in leading to unrestricted cellular proliferation. Moreover, the abilities of E6 and E7 proteins of different HPV types to bind the oncosuppressors appear to reflect their oncogenic potentials, in that HPV types 16 and 18 E6 and E7 proteins bind with greater affinity than those of HPV 6 or HPV 11 (Munger et al., 1989; Werness et al., 1990). In support of this, analysis of a series of HPV positive and negative human cervical carcinoma cell lines for mutation of the p53 and Rb genes found that the HPV positive cell lines contained normal Rb and low levels of wild-type p53, whereas in cell lines lacking HPV DNA mutations were identified in both genes. Interestingly, the mutations in p53 were located in highly conserved regions where mutations appear in a variety of human cancers (Scheffner et al., 1991). These results indicate the importance of inactivation of the two oncosuppressor proteins Rb and p53 in the development of cervical cancer.

1.6 Regulation of HPV Gene Expression

1.6.1 Organisation of the URR

The HPV URR is approximately 1000 nucleotides long and lies between the L1 and E6 genes. The 5' end of the URR is rich in poly AT/GT sequences. Such sequences frequently found in the human genome are capable of forming Z-DNA structures and enhancing gene expression

(Hamada et al., 1982, 1984), however their function in the HPV life cycle is not understood. The 3' region of the URR contains the E6 promoter (figure 1.5). Between the AT/GT rich region and promoter elements lies a variety of binding sites and enhancer elements some of which are responsive to the viral E2 proteins and others which are responsive to cellular factors.

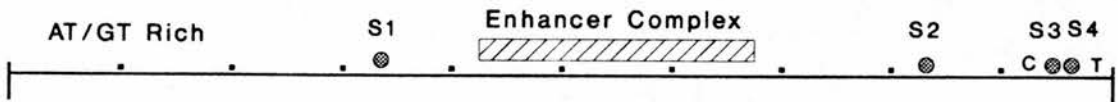
1.6.2 Regulation by E2

All the PVs sequenced to date have been found to have E2 binding sites in the URR (Dartmann et al., 1986), however these sites differ considerably in number, position, E2 responsiveness and conservation of the palindrome (which determines the binding affinity of the E2 proteins). All the genital HPVs studied have four E2 binding sites arranged in a similar manner within the URR (figure 1.5) Two sites (S3 and S4) are situated between the CAAT and TATA boxes of the putative E6 promoter and correspond to the perfect palindromic sequence. A single site situated another 100 nt upstream (S2) is a perfect palindrome in HPV 6 and 11 but in HPV 18 and HPV 16 this site corresponds to the degenerate palindromic sequence. Finally, another single site situated a further 400 nt upstream (S1) is a perfect palindrome.

The contribution of each of the E2BS to overall E2 dependent enhancer activity has been investigated in several studies. Site 1 was originally thought to play no role in E2 dependent enhancer activity (Phelps and Howley, 1987; Hirochika et al., 1988; Gius et al., 1988) however footprinting assays on HPV 18 have found this site to be E2 protected (Garcia-Carranca et al., 1988) and mutational analyses on HPV 18 (Thierry et al., 1990) and HPV 11 (Broker et al., 1990) have indicated a

Figure 1.5

Organization of the URR of the common genital HPV types. The 5' terminus of the URR contains a region rich in AT/GT sequences. A central enhancer complex is activated by binding of cellular factors. Shaded circles represent binding sites (S1-S4) for the various E2 proteins. C and T represent the CAAT and TATA boxes of the E6 promoter (Hirochika et al., 1988; Chong et al., 1990).



possible role for this site in the positive regulation of gene expression. Site 2 is a perfect palindrome in HPV 11 and forms part of an E2 responsive enhancer (Hirochika et al., 1988; Chin et al., 1989) whereas in HPV 18 this site is a degenerate palindrome and is not footprinted by E2 (Garcia-Carranca et al., 1988). Despite this, recent studies suggested that this site can contribute to E2 dependent negative modulation of gene expression in HPV 18 (Thierry and Howley, 1990).

When the region containing sites 3 and 4 is cloned with the E6 promoter in its natural context, transcription is repressed by the full length E2 protein (Thierry and Yaniv, 1987; Bernard et al., 1989; Chin et al., 1989). Since these 2 sites are situated very close to the E6 promoter between CAAT and TATA boxes, repression may be due to steric interference by E2 with the binding of transcription factors such as TFIID or RNA polymerase. In contrast, when cloned in an unnatural context, upstream of a heterologous promoter, the region containing sites 3 and 4 acts as a transcriptional enhancer in response to the full length E2 protein. Thus it would appear that the full length E2 protein can act either as a transactivator or a repressor depending on the location of the E2 binding sites in relation to the promoter (Thierry and Yaniv, 1987; Bernard et al., 1989). Furthermore, recent studies have found that the concentration of E2 protein present is an important factor in determining whether E2 acts as a transcriptional repressor or a transactivator. It was suggested that E2 may act as a repressor at high concentration and as a transcriptional transactivator at low concentration thus regulating its own transcription and that of E6 and E7 (Broker et al., 1990).

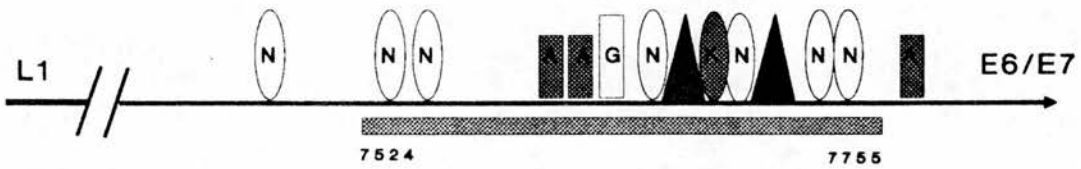
1.6.3 Regulation by cellular factors

Cellular hybrids of the aneuploid cervical carcinoma cell line HeLa, which contains HPV 18, and normal human fibroblasts lose both tumorigenicity in nude mice (Stanbridge et al., 1982) and expression of HPV 18 *in vivo* (zur Hausen, 1986). Hybrids progressively lose chromosomes and revertants that lose chromosome 11 regain HPV gene expression and tumorigenicity (zur Hausen, 1986). On the basis of this evidence it has been suggested that there may be a gene on chromosome 11 coding for a cellular interfering factor (CIF) which suppresses HPV gene expression (zur Hausen, 1986). In support of this the homologous enhancer/promoter of HPV 16 functions much more strongly in cells which lack the short arm of chromosome 11 (Smits et al., 1990).

The HPV URR has been found to contain binding sites for a variety of cellular factors including nuclear factor 1 (NF1), activator protein 1 (AP1) and steroid receptors (a glucocorticoid responsive element (GRE))(figure 1.6). Transcription studies on isolated fragments of the HPV 16 URR have found that individual sites have little intrinsic enhancer activity, but may act synergistically when combined (Chong et al., 1990)(figure 1.6). The precise role of the GRE in HPV transcriptional control is unknown but it seems likely that it confers some advantage on the virus since it has been maintained through evolution in many divergent HPV types. The GRE has been shown to mediate responsiveness to progesterone. Genital HPV types frequently infect epithelial cells bearing progesterone receptors. Thus, it is likely that viral gene expression would be increased in these cells during the female ovarian cycle and more extensively during pregnancy when warts and some cervical cancers have been observed to grow rapidly (Chan et al.,

Figure 1.6

Organization of cellular factor binding sites along the HPV 16 URR. A fragment reported to contain enhancer activated is underlined with a shaded bar. Binding sites for nuclear factor I (N), activator protein 1 (A), glucocorticoid responsive elements (G), novel factors - papillomavirus associated factor and nuclear factor associated (solid triangles) are shown. K denotes the core sequence of the keratinocyte dependent enhancer. The other common genital HPV types have a similar arrangement. Data redrawn from Chong et al (1990).



1989).

A keratinocyte dependent enhancer activity has been identified in the HPV 16 URR (figure 1.6). Essential to this activity is a core sequence TTNGGTTT (Cripe et al., 1987) found in all the genital HPVs sequenced so far (Dartmann et al., 1986). The inverted consensus sequence AANCCAAA is present upstream of several human cytokeratin genes (Blessing et al., 1987). It has been suggested that the viral element responds to the same cellular factors as these keratinocyte specific genes.

The effects of specific cellular factors involved in cellular differentiation on HPV gene expression were studied by Woodworth et al (1990). Cellular transforming growth factors beta 1 and 2 were found to down regulate HPV 16 transcription in immortalized non-tumorigenic cervical cells. Prolonged culture *in vitro* or malignant transformation of these cells resulted in partial resistance to this inhibitory effect on HPV transcription which varied according to the degree of cellular differentiation. Epidermal growth factor (EGF) down regulated HPV 16 E6/E7 expression in an immortalized human keratinocyte cell line. An EGF responsive element has been identified and found to overlap the region previously mapped to the keratinocyte dependent enhancer (Yasumoto et al., 1991).

1.7 Alterations in the Viral Upstream Regulatory Region (URR)

Viral variants with sequence alterations within the URR have been found in genital carcinomas and unusual lesions and it has been suggested that as these variants are likely to have an altered pattern of gene expression, they may contribute to the neoplastic process.

There are several reports of sequence alterations in the URR of the low risk HPVs. Sub types HPV 6vc and HPV 6T-70 both isolated from invasive vulvar carcinomas had deletions and/or insertions in the 5' AT/GT rich region of the URR (Rando et al., 1986; Kasher and Roman, 1988). It was proposed that the duplication of these sequences in the URR might be sufficient for conversion to a malignant phenotype on the basis that similar sequences found in the human genome are capable of enhancing gene expression. However, a subsequent study showed that HPV 6T-70 and an HPV 6 isolate from a benign lesion had similar relative enhancer activities and transforming potentials (Farr et al., 1991). While recent reports describe DNA-protein binding interactions and enhancer activity in the 5' end of the HPV 11 URR it is still difficult to ascertain the significance of sequence alterations within this region (Auborn and Steinberg, 1991).

HPV 6ma was cloned from a condyloma of the nipple. This isolate contained a 236bp duplication of sequences in the 3' region of the URR incorporating an NF1 site, the cytokeratin octamer and an E2 binding site. Interestingly a fragment containing the entire duplication was found to have increased enhancer activity. It was suggested that this duplication may influence early gene expression and possibly tissue tropism (Kulke et al., 1989).

Boshart and zur Hausen (1987) described another subtype of HPV 6 - HPV 6d - which they isolated from a Buschke-Lowenstein tumour. This subtype differs from HPV 6b by a 459bp duplication of the 3' end of the URR. This duplication contained many of the putative control elements

for early gene transcription which the authors suggest may influence the biological potential of that virus. The first report of a tandem duplication of the entire URR was of an HPV 11 isolate found in a lung metastasis from a patient with chronic laryngotracheobronchial papillomatosis (Byrne et al., 1987).

Until recently there have been relatively few reports of sequence alterations in the URR of the high risk HPV types. The first indication that viral variants may be transmissible came from Tidy et al (1989) who found two independently derived cervical carcinomas infected with an episomal variant of HPV 16. This variant had a deletion of a large portion of the 3' end of the URR which included the GRE, the keratinocyte dependent enhancer, E2 binding sites, a CAAT and a TATA box. Preliminary reports describe four further variants of HPV16, with sequence alterations in the 5' AT/GT rich region of the URR, found in cases of vulvar bowenoid papulosis, vulvar condyloma and early vulvar carcinoma (Fang et al., 1990; Donghi et al., 1990).

1.8 Physical state of HPV DNA in Cervical Tumours

Another mechanism which could alter the normal pattern of viral gene expression and thus contribute to the neoplastic process is the integration of HPV DNA into the host genome. Papillomavirus DNA is well known to exist as an extrachromosomal episome in benign cervical lesions whereas, in most human cervical carcinomas and carcinoma-derived cell lines HPV 16 and 18 have been found integrated into the human chromosome (Boshart, 1984; Durst et al., 1985; Pater and Pater, 1985; Shirasawa et al., 1987; Tidy et al., 1989). In one study of 100 HPV positive CIN lesions, only 3 showed integrated HPV DNA and these

were higher grade dysplasias. In contrast, the same study detected integrated HPV DNA in 81% of 69 cervical carcinomas (Cullen et al., 1991). Integration of high risk HPV types has also been shown to occur in experimental models. Human genital keratinocytes immortalized by HPV types 16, 18, 31 or 33 contain integrated and transcriptionally active viral genomes whereas HPV 6 or 11 transfected cells are not immortalized and contain only extrachromosomal viral DNA (Woodworth et al., 1989).

1.8.1 Effects of integration on the viral genome

In the viral genome, the integration event most often disrupts the E1 or E2 ORFs leading to the functional inactivation of the E2 gene which encodes positive and negative regulatory functions for the viral promoter element in the URR (Choo et al., 1987; Lambert et al., 1987b; Cripe et al., 1987). While other viral genes and functions may be lost through focal deletion or decoupling from the URR, the E6 and E7 transforming genes and the URR are invariably retained (Pater and Pater, 1985; Schwarz et al., 1985; Baker et al., 1987). These observations have led to speculation that disruption of the E2 repressors may allow over-expression of the E6 and E7 oncoproteins, promoting the development of neoplasia (Choo et al., 1987). Viral integration within E1/E2 region could also mean the loss or uncoupling of the usual viral signals which terminate transcription. Thus transcription of E6 and E7 genes would run on into adjacent host DNA until terminated by host signals. In theory, this could give rise to an E6/E7 coding mRNA with increased stability or translatability possibly leading to increased levels of E6/E7 proteins (Arends et al., 1990).

1.8.2 Effects of integration on the cell genome

The cause of viral integration is unknown but it has been suggested that it may be induced by carcinogens or due to intrinsic properties of the virus. The monoclonal pattern of viral integration indicates that this occurs prior to expansion of the malignant clone of cells.

While there is no unique site within the host genome at which viral integration occurs, evidence that it is not an entirely random process has come from a study by Choo et al (1990) who showed that HPV 16 integration in a cervical carcinoma had been guided or influenced by short regions of sequence homology within the viral genome and cellular DNA. Furthermore, fragile sites and proto-oncogene locations appear to be preferential targets for viral integration (Popescu and DiPaolo, 1989). The significance of this event is discussed below.

1.9 HPV and Cellular Oncogenes

In HPV-associated cervical cancers the results of several studies indicate the probable involvement of cellular oncogenes. In a series of HPV 16 or 18 positive cervical carcinomas amplification of oncogenes *c-myc* and *c-Ha-ras* was correlated with advanced tumour stage (Riou et al., 1984). Overexpression of *c-myc* was also correlated with poor prognosis for early stage invasive carcinomas with an eight fold greater incidence of early relapse compared to those with normal *c-myc* expression (Riou et al., 1987). A further study found that a high proportion of HPV 16 or 18 positive cervical carcinomas with mutated or deleted *c-Ha-ras* genes also exhibited overexpressed or amplified levels of *c-myc* suggesting possible complementation between these two oncogenes in tumour progression (Riou et al., 1988).

Interestingly, studies on the integration sites of HPV 18 in HeLa and C4-1 cervical carcinoma cell lines found that the virus had inserted within 40 kb upstream of the *c-myc* gene and that the level of *c-myc* mRNA was increased in both cell lines raising the possibility that HPV integration caused *cis*-activation of *c-myc* (Durst et al., 1987b). The influence of HPV integration on nearby proto-oncogenes or other genes involved in cellular differentiation remains to be examined.

1.10 Additional Risk Factors for Cervical Neoplasia

Cigarette smoking, oral contraceptives, Herpes Simplex Virus (HSV) infection and immune status have all been suggested as possible risk factors for the development of cervical cancer. Epidemiological studies have found a low but consistent increase in relative risk due to heavy or prolonged smoking and a probable increase from prolonged use of oral contraceptives. Tobacco metabolites and mutagenic activity have been detected in the cervical mucus of smokers indicating the opportunity for a direct carcinogenic effect (Brinton and Fraumeni, 1986).

Herpes simplex virus has the potential to act as a hit and run initiator and recent studies have found that HSV-2 is capable of converting HPV-16 immortalized human genital keratinocytes to tumorigenicity (DiPaolo et al, 1990). However, a prospective investigation by Vonka et al (1984) revealed no relationship between HSV-2 infection and subsequent neoplasia among a cohort of women.

An increased risk of HPV infection and CIN has been observed in renal allograft patients (Alloub et al., 1989) who are immunosuppressed.

Recurrence of condylomata and CIN appears to be influenced by immune suppression resulting from various causes (Porecco et al., 1975; Sillman et al., 1984; Halpert et al., 1986). A recent study found a high risk of cervical carcinoma for women with HLA-DQw3, a histocompatibility antigen (Wank and Thomssen, 1991), suggesting a role for the immune response genes.

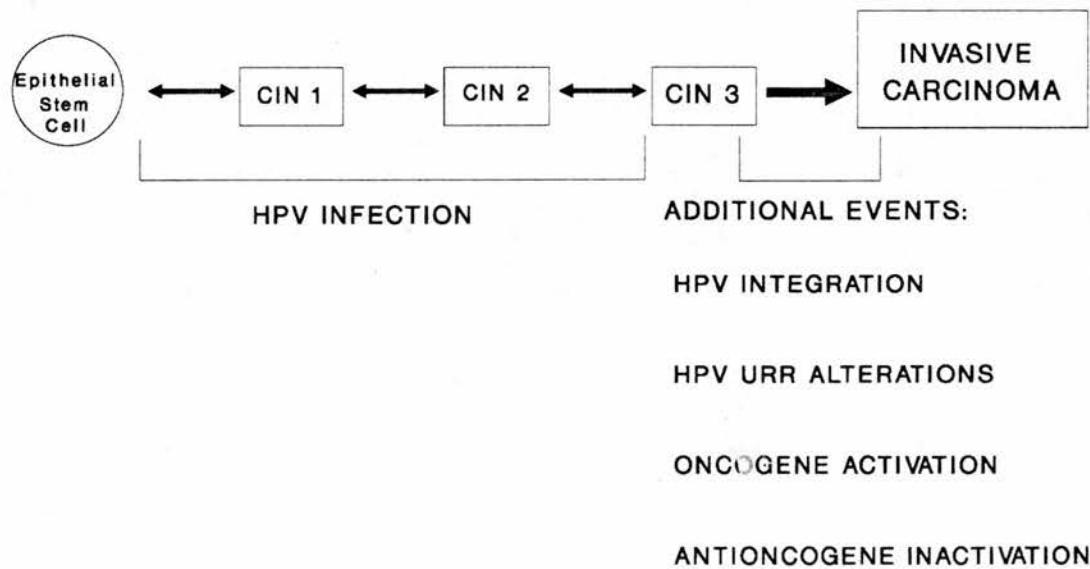
1.11 A Multistage Model Cervical Carcinogenesis

The current view of cervical neoplasia is that of a multistage disease which develops sequentially from CIN 1 through CIN 3. All CIN lesions are potentially reversible. Further progression to invasive carcinoma represents a qualitatively different and irreversible change. Epidemiological studies show a clear correlation between HPV infection and cervical neoplasia and the oncogenic potential of the virus has been demonstrated in many transformation studies. However, HPV infection does not appear to be the only factor involved in the development of cervical cancer, as many cases of HPV infection do not develop into cervical cancer. In keeping with the multistage model for cervical carcinogenesis additional events or factors are thought to be required for neoplastic progression.

A model of events which may occur in development of cervical cancer is shown in figure 1.7. In this model infection of epithelial stem cells with HPV provokes intraepithelial neoplasia presumably due to the interaction of viral proteins and host cell-responsive DNA sequences in maturing epithelia.

Figure 1.7

A model of possible events contributing to the development of cervical cancer. HPV infection of basal stem cells in the cervical epithelium, perhaps aided by co-factors provokes CIN. HPV variants with URR alterations or the integration of HPV DNA may contribute to neoplastic progression through an altered pattern of viral gene expression. Inactivation of oncosuppressors p53 and Rb and oncogene activation may also play a role in conversion to invasive carcinoma.



Subsequent progression may involve an altered pattern of gene expression such that the viral E6 and E7 transforming genes are expressed to a high level. Integration of HPV DNA of the high risk types into the host genome has the potential to alter viral gene expression in this way. The integration event results in conservation of intact URR/E6/E7 DNA sequences and inactivation or loss of expression of E2 which encodes regulatory proteins for viral transcription and L1 and L2 which may provide targets for an immune response. Alternatively, HPV variants of high or low risk type with alterations to the URR may have an altered pattern of gene expression such that E6 and E7 are overexpressed. Evidence exists to support degradation and functional inactivation of oncosuppressor proteins p53 and Rb by the E6 and E7 proteins of the high risk HPV types. Loss of control of viral gene expression by the putative CIF genes on chromosome 11 may also contribute to the neoplastic process by allowing continuous expression of viral genes. Activation of the *c-myc* and *c-Ha-ras-1* oncogenes may play a role: *myc* activation appears to correlate with early stage tumours and *ras* activation with late stage tumours.

While this model for cervical carcinogenesis explains some of the events likely to be involved in the development of invasive carcinoma there are many aspects which require further investigation and clarification. In order to establish a role for HPV in cervical cancer it is important to have some indication as to the prevalence of the virus in the normal population. Reports on the prevalence of HPV DNA in the normal population vary widely and some investigators have found a high prevalence of HPV 16 in their control cases casting doubt on the role of HPV in cervical cancer (Schneider et al., 1987; Young et al., 1989).

These widely varying rates are most likely to be a consequence of the different detection or cervical sampling methods used or the source of the control subjects (Munoz et al., 1988). Application of sensitive detection assays to focussed cervical samples from control groups representative of the normal population should help to clarify this matter.

Whether the usual pathway of progression from HPV infection to invasive cancer is sequential from CIN 1 via CIN 2 and CIN 3 or direct from any of these to cancer is not known. Few investigators appear to have addressed this question and consequently there is little accurate data on HPV prevalence at each stage of the disease.

The common HPV types are believed to differ in oncogenic potential and thus have been assigned differing levels of risk. HPV 6 and 11 appear to confer a low risk and HPV 16 and 18 a high risk of malignant transformation. Possible differences in behaviour of associated tumours may exist amongst the high risk HPV types with HPV 18 more prevalent in glandular than squamous cervical carcinomas and having a greater tendency toward rapid progression. Further studies aimed at generating accurate data on the prevalence of individual HPV types in CIN, squamous and glandular carcinomas may prove useful in substantiating these findings.

In recent years HPV variants of both high and low risk types with large scale alterations to the URR have been discovered. While it has been suggested that lesions containing these variants may have a greater tendency toward neoplastic progression little is known of their overall prevalence in CIN or cervical carcinomas. Integration of high risk type

HPV DNA into the host genome is thought to play an important role in neoplastic progression. However the precise stage in cervical carcinogenesis at which this event occurs is unclear. Investigation of both the prevalence of HPV variants in CIN and carcinoma and the stage in the CIN-cancer sequence at which integration usually occurs will require the design of new sensitive assays which are applicable to small CIN biopsies.

1.12 Aims

This study aimed to establish a more precise role for HPV in the development of cervical cancer and also to determine the key stages in the neoplastic process at which HPV contributes. The approach was to correlate the presence of specific high and low risk HPV types, sequence alterations within the HPV URR, and the physical state of HPV DNA, with the grade of CIN and invasive carcinoma.

CHAPTER 2

MATERIALS AND METHODS

2.1 Clinical material

Sixty cervical biopsy specimens were obtained from women referred for diagnosis or treatment because of abnormal smears to Elsie Inglis Colposcopy Clinic, Edinburgh. Abnormal aceto-white cervical lesions detected colposcopically were sampled by punch biopsy and immediately snap frozen in liquid nitrogen. Adjacent sections were taken for HPV detection and histological assessment. Control cervical blocks were taken at forensic autopsy from young women (within 24 hours of accidental death) courtesy of Professor Busuttil, Forensic Medicine Unit, Department of Pathology, Edinburgh. Two transverse sections were taken from the transformation zone of the control cervixes, one was immediately snap frozen in liquid nitrogen, the other was fixed in neutral buffered formalin and processed as a paraffin block for histological assessment. Adjacent sections of the frozen tissue were taken for HPV detection and additional histological assessment.

Formalin-fixed paraffin-embedded tissues of 26 cases of squamous carcinoma, 16 cases of adenocarcinoma and 4 cases of adenosquamous carcinoma diagnosed as part of the routine diagnostic service were chosen from the file of routine surgical specimens of the University of Edinburgh. The specimens were cone biopsies or hysterectomies performed between 1982 and 1990.

2.2 Histopathological Examination

Tissue sections from all specimens were stained with haematoxylin-eosin. Every case of carcinoma was also stained with Alcian blue/periodic acid Schiff. Histology of all specimens was assessed independently by two experienced pathologists - Dr M Arends and Dr E

Duvall - according to standard criteria (Ferenczy and Winkler, 1987). Grade of CIN was diagnosed on the basis of whether abnormal cells filled one third, two thirds or the entire thickness of the epithelium, independent of koilocytic change. In a small number of the CIN cases (equally distributed amongst CIN 1, CIN 2 and CIN 3) final histological assessment was resolved by consensus. The diagnosis of squamous carcinoma was on the basis of evidence of squamous differentiation, either keratinization or prickles formation. Glandular carcinoma was diagnosed on the basis of mucin secretion and formation of glandular structures. Adenosquamous carcinoma was diagnosed in cases which showed a mixed pattern: at least 10% squamous and 10% glandular differentiation. Normal exocervical and endocervical epithelium and glands were identified in all the tissue sampled at autopsy.

2.3 Oligonucleotide Selection and Synthesis

HPV DNA sequences, derived from the EMBL genetic sequence database, were analysed for the selection of PCR primers and oligoprobes using the University of Wisconsin (UW) Genetics Computer Group Software (Cameron, 1988; Devereux et al., 1984). Criteria on which selection was made are explained in the relevant chapters. Oligonucleotides suitable as primers and probes were synthesized on an Oswel Gene Synthesiser (Dept. of Chemistry, University of Edinburgh, UK) and were HPLC purified. During synthesis a biotin moiety, on a 15 carbon atom linker arm, was added to the 5' end of the oligonucleotide probes.

2.4 Preparation of Materials for PCR

Two 20um **frozen sections** were cut from punch biopsies of cervical

intraepithelial neoplasia or normal cervix. The sections were briefly thawed on the surface of ice-chilled distilled water in a 1.5ml Eppendorf tube. Concentrated PCR solution (detailed below) was added to give a final volume of 100ul and this was heated to 98°C for 10 min prior to amplification. Heat denaturation for longer periods was tested but no difference in efficiency of PCR was observed.

Two 20um sections cut from **paraffin blocks** of cervical carcinoma were placed in a 1.5ml Eppendorf tube, suspended in 200ul digestion buffer (100mM NaCl, 10mM Tris-Cl, 25mM EDTA, 0.5% SDS, pH 8.4) containing 0.1mg/ml proteinase K and incubated at 37°C for five days. Nucleic acid was purified by an organic extraction step. An equal volume (200ul) of phenol:chloroform:iso-amyl alcohol (25:24:1) was added to the proteinase K digests, mixed and centrifuged to separate the aqueous and organic layers. The upper aqueous layer was collected and the extraction repeated. A final extraction was carried out using an equal volume of chloroform:iso-amyl alcohol (24:1). The nucleic acid was then precipitated with one half volume (100ul) 7.5M ammonium acetate and two volumes (600ul) of cold ethanol at -20°C for 16-20 hours, spun down (1300 rpm, for 15 minutes), vacuum dried and dissolved in 20ul TRIS-EDTA buffer for three days. 98ul of PCR solution was added to 2ul of extracted nucleic acid prior to amplification.

The **cultured cells**, HeLa, SiHa and Raji, were obtained from the American Type Culture Collection and grown *in vitro* in Dulbecco's MEM medium with 10% heat inactivated foetal calf serum by standard methods. Up to 10⁴ cells were suspended in 20ul phosphate buffered saline (PBS) and prepared for PCR by boiling for 15 min. 80ul

concentrated PCR solution was added just prior to amplification.

2.5 Polymerase Chain Reaction

The PCR solution consisted of reaction buffer (50mM KCl, 10mM Tris-HCl (pH8.3 at room temperature), 1.5mM MgCl₂, 0.01% gelatin), 200uM of each dNTP, 1.0uM of each primer, 2-2.5 units Taq Polymerase. PCR solution was added to template to give a final volume of 100ul and overlaid with 100ul mineral oil. Samples were subjected to 30-35 cycles of PCR on an automated heating block (Hybaid), each cycle consisting of DNA duplex denaturation at 94°C for 1 min, primer annealing at 45-55°C for 2 min and DNA synthesis by primer extension at 72°C for 3 min. During the final cycle, the extension step lasted 10 min. The reaction product was electrophoresed on a 1%-4% agarose (Nusieve GTG, Seakem GTG) gel containing 2ug/ml ethidium bromide in Tris-Borate buffer (Kodak) along with a DNA size marker, and visualised under UV light.

2.6 Procedures to Minimise the Risk of Contamination

Stringent precautions were taken to minimise the risk of contamination of the PCR solution with HPV DNA from unwanted sources. Tissue sections from different cases were cut using either separate microtome blades or previously unused regions of the same blade (when taking sections from small punch biopsies). Sections cut from a block of tissue not containing HPV (eg myocardium) were included where appropriate, to investigate the possibility of transfer of HPV DNA from case to case, during section cutting or proteinase K digestion. Separate laboratories were used for preparation of the PCR solution, amplification in the automated heating block, and analysis of the amplified DNA by gel

electrophoresis or dot blot hybridisation. The components of the PCR solution were prepared in small aliquots prior to use, and added to the reaction in a class II biological safety cabinet, using positive displacement pipettes exclusively dedicated to this purpose. Whilst setting up the reactions the operator adopted a microbiological standard sterile technique and wore disposable gloves.

2.7 Amplification of Reference Gene

All cases found to be PCR negative for the 5 HPV types were checked for amplifiable DNA and absence of Taq polymerase inhibition using a separate set of specific primers for a reference gene c-Ha-ras (primer 1, 5'GACGGAATATAAGCTGGTGG3'; primer 2, 5'TGGATGGTCAGCGCACTCTT3') (Verlaan de-Vries et al., 1986). The amplified products of all cases found to be HPV positive in the PCR assay were subjected to confirmation by dot blot hybridisation as described below.

2.8 Dot Blot Hybridisation

Nitrocellulose filters were pretreated with distilled water followed by 15 X SSC (standard sodium citrate). The amplified DNA was resuspended in 20ul TE buffer and serially diluted ten- and one hundred-fold in 15 X SSC. 10ul samples, made up to a final volume of 100ul in 15 X SSC, were subjected to heat denaturation at 98°C for 5 min, and vacuum blotted onto nitrocellulose filters using "Hybridot" manifold (Gibco-BRL). The sample wells were washed through with a further 100ul 15 X SSC.

The filters were baked at 80°C for 2h and prehybridised for 1h at 42°C in 5 X SSC, 25mM NaH₂PO₄ (pH6.5), 5 X Denhardts solution, 0.1% sodium dodecyl sulphate (SDS) and 200ug/ml denatured salmon sperm

DNA. The prehybridisation solution was replaced with the hybridisation solution which contained the same components with the addition of 0.8uM biotinylated oligonucleotide probe (heated to 98°C for 10 min before use to denature any secondary structure), and differing concentrations of deionised formamide optimized for the different probes (0%, 10%, 15%, 20% and 0% for HPV types 6, 11, 16, 18 and 33 respectively)(Albretsen et al., 1988). Filters were hybridised at 42°C for 16-20h, washed twice at 20°C in 2 X SSC with 0.1% SDS, twice at 42°C in 0.5 X SSC with 0.1% SDS and baked at 80°C for 1h.

Bound, biotinylated probe was detected using a modification of the "Blugene" protocol (Gibco-BRL). Non-specific streptavidin binding sites were blocked by incubating the filters for 20 min at 42°C in a solution of 0.1M Tris-HCl (pH7.5), 0.1M NaCl, 2mM MgCl₂, 0.05% Triton X-100 (buffer 1) with bovine serum albumin (Sigma) added to a final concentration of 3% (buffer 2). The filters were baked at 80°C for 1h, rehydrated in buffer 2 for 10 min, incubated at 20°C for 10 min in 2ug/ml streptavidin solution (Gibco-BRL), washed twice in buffer 1, and incubated at 20°C in 1ug/ml biotinylated alkaline phosphatase solution (Gibco-BRL) for 10 min. The filters were then washed twice in buffer 1 at 20°C for 10 min and twice in 0.1M Tris-HCl (pH9.5), 0.1M NaCl, 50mM MgCl₂ (buffer 3) at 20°C for 10 min. Bound alkaline phosphatase was visualised by incubation in the dark, at 20°C in a solution of 0.33mg/ml nitro-blue tetrazolium (NBT) and 0.166mg/ml 5-bromo, 4-chloro, 3-indolyl phosphate (BCIP) in buffer 3. After 1-2h, deposition of the blue-purple coloured reaction product was terminated by immersing the filters in 20uM Tris (pH7.5), 5mM EDTA and the filters were baked at 80°C for 1-2 min.

2.9 Restriction Enzyme Mapping

Amplified DNA products were concentrated by ethanol precipitation and resuspended in 20ul restriction enzyme buffer. These were digested with appropriate enzymes at 37°C for 16-20h. 10ul DNA solutions of both HPV 6b and 11 were restricted with DdeI (22 units)(Sigma), HPV 16 and 18 with HinfI (20 units)(Sigma) and HPV 33 with DraI (24 units)(Amersham). The digested DNA was subjected to electrophoresis at 50v for 4h on a 2.5% agarose gel in Tris-Borate buffer (Kodak)

2.10 Anchor PCR

10ug cloned HPV 16 plasmid DNA was digested with AatII (16 units) (BCL) in a total volume of 20ul at 37°C for 16-20h in triplicate reactions. The DNA from one reaction was electrophoresed at 50v on a 1% agarose gel to ensure digestion had been complete.

DNA from the second reaction was used to check the efficiency of the 3' tailing reaction: digested DNA was tailed with terminal deoxynucleotidyl transferase (TdT)(15 units)(Gibco-BRL) in 40ul reaction mixture containing tailing buffer (2mM CoCl₂, 0.1M potassium cacodylate (pH 7.2), 200uM DTT)(Gibco-BRL), 2.5uM dATP and 4ul [P³²] dATP (10 mCi/ml, >400 Ci/mmol) for 1h at 37°C. Reaction products were electrophoresed on a 1% agarose gel which was then exposed to X-ray film for 16-20h at 20°C sealed in a light tight cassette.

Digested DNA from the remaining reaction was tailed with TdT (15 units) in a 40ul reaction mixture containing tailing buffer (as above) and

2.5uM dATP for 1h at 37°C. For anchor-PCR, 10ul of tailed DNA was added to a reaction mixture containing 50mM KCl, 10mM Tris-HCl (pH8.3 at room temperature), 1.5mM MgCl₂, 0.01% gelatin (PCR buffer), 200uM of each dNTP, 1.0uM adapter primer, 1.0uM HPV specific primer, 0.2uM hybrid primer to give a final volume of 100ul. The mixture was then overlaid with paraffin oil to prevent evaporation and incubated at 94°C for 5 min for DNA denaturation. After cooling to 72°C, 2.5 units of Taq Polymerase (NBL) was added. To allow for the annealing of the hybrid primer to poly A tails and subsequent extension, the first cycle of amplification was annealed at 55°C for 5 min and extended at 72°C for 40 minutes. The following 40 cycles of amplification were denatured at 94°C for 1 min, annealed at 55°C for 2 min and extended at 72°C for 4 min (with the exception of the final cycle which was extended for 15 min). 20ul of the final reaction products was electrophoresed on a 1% agarose gel (Seakem GTG) containing 2ug/ml ethidium bromide in Tris-Borate buffer (Kodak) at 75v for 2h with a DNA size marker - the 1kb ladder (fragment sizes: 12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,084; 2,036; 1,635; 1,018; 516/506; 394; 344; 298; 220; 200; 154; 142; 75)(Gibco- BRL).

CHAPTER 3

DESIGN AND OPTIMIZATION OF A PCR DETECTION ASSAY

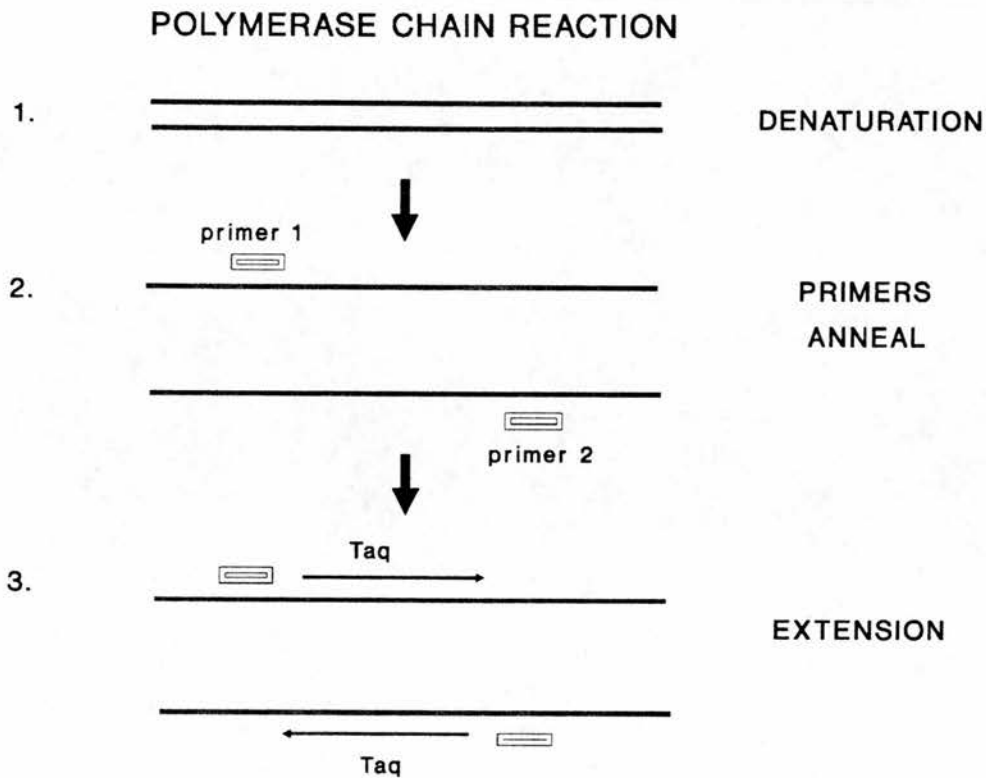
3.1 Introduction

A variety of techniques have been used to detect HPV in clinical samples. Until recently most studies used hybridisation techniques such as Southern blot, tissue *in situ*, dot blot and filter *in situ* (FISH) hybridisation (Lorincz et al., 1987; Tase et al., 1988; Pater et al., 1986; de Villiers et al., 1987). Southern hybridisation is a sensitive method of detecting gene sequences but it is expensive, time consuming, labour intensive and requires relatively large quantities of fresh tissue. Tissue *in situ* hybridisation allows visualisation of target DNA sequences in specific cells but can lack sensitivity and prolonged incubation times mean that the technique usually takes days to perform. The FISH and dot blot techniques are less laborious but are of low specificity and sensitivity.

A recently developed technique, polymerase chain reaction (PCR), is capable of amplifying very small amounts of specific DNA sequences within hours to a level detectable by gel analysis. The PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridise to opposite strands and flank the region of interest in the target DNA. Repetitive cycles of template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase results in the near exponential accumulation of a specific DNA fragment whose termini are defined by the 5' ends of the primers (figure 3.1). Because the products of one cycle can serve as a template in the next, the number of target DNA copies approximately doubles at every cycle. This highly sensitive technique has been used to identify one copy of HPV DNA in 10^5 cells (Young et al., 1989) and has been successfully applied to tissue which has been formalin fixed and

Figure 3.1

The standard PCR reaction involves repeated cycling of three steps: (1) denaturation at 94°C, (2) primer annealing at between 42°C and 60°C dependent upon primer constraints, (3) extension of annealed primers by Taq polymerase at 72°C. The time for each step is specific to the system used. Repeated cycling of these three steps results in near logarithmic amplification of a specific DNA fragment.



paraffin wax embedded for histological examination (Shibata et al., 1988)

In this study, a detection assay was designed which involved PCR amplification of HPV DNA using primers specific for each HPV type. Amplified products were identified by gel electrophoresis and separately by hybridisation with oligoprobes specific for each HPV type. The assay was optimized for absolute HPV type specificity, sensitivity and applicability to tissue from different sources.

3.2 Results

3.2.1 Design of oligonucleotides for the HPV detection assay

Several factors were taken into account when designing primers and probes for the HPV detection assay. Target sequences for oligonucleotide binding had to be conserved following integration and could therefore only be chosen from the URR, E6 or E7 ORFs (figure 3.2). As oligonucleotide sequences had also to be unique to each HPV type for the assay to be HPV type specific, the E6 and E7 genes were searched for regions of HPV type specific sequence divergence.

To distinguish between the most similar HPV types, the University of Wisconsin (UW) computer program COMPARE was used. The E6 genes of HPV 6b and 11, HPV 16 and 18, HPV 16 and 33 and HPV 18 and 33 were compared. Using the UW program DOTPLOT, regions with dissimilar sequences appear as gaps in the diagonal line of colinear homology in the dot plots (figure 3.3). To examine these dissimilar sequences at nucleotide level, the UW GAP program was used. This program allows the determination of the exact extent of base mismatching of the most similar HPV types. For each HPV type, three

Figure 3.2

Pattern of integration of HPV DNA by disruption of the circular viral genome at the E1-E2 region in three cervical carcinoma cell lines (SW756, HeLa, C4-1). The transforming genes E6 and E7, adjacent to the viral promoters and enhancers in the upstream regulatory region remain intact, whilst other viral coding sequences are not always retained. Solid lines represent host cell DNA. The target sequence (hatched box) for amplification by PCR, directed by HPV type-specific primers (arrows), lies within the E6 gene. Data redrawn from Schwarz et al (1985).

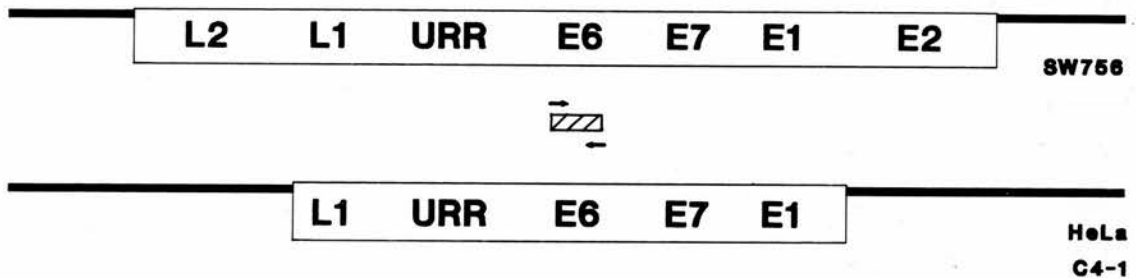
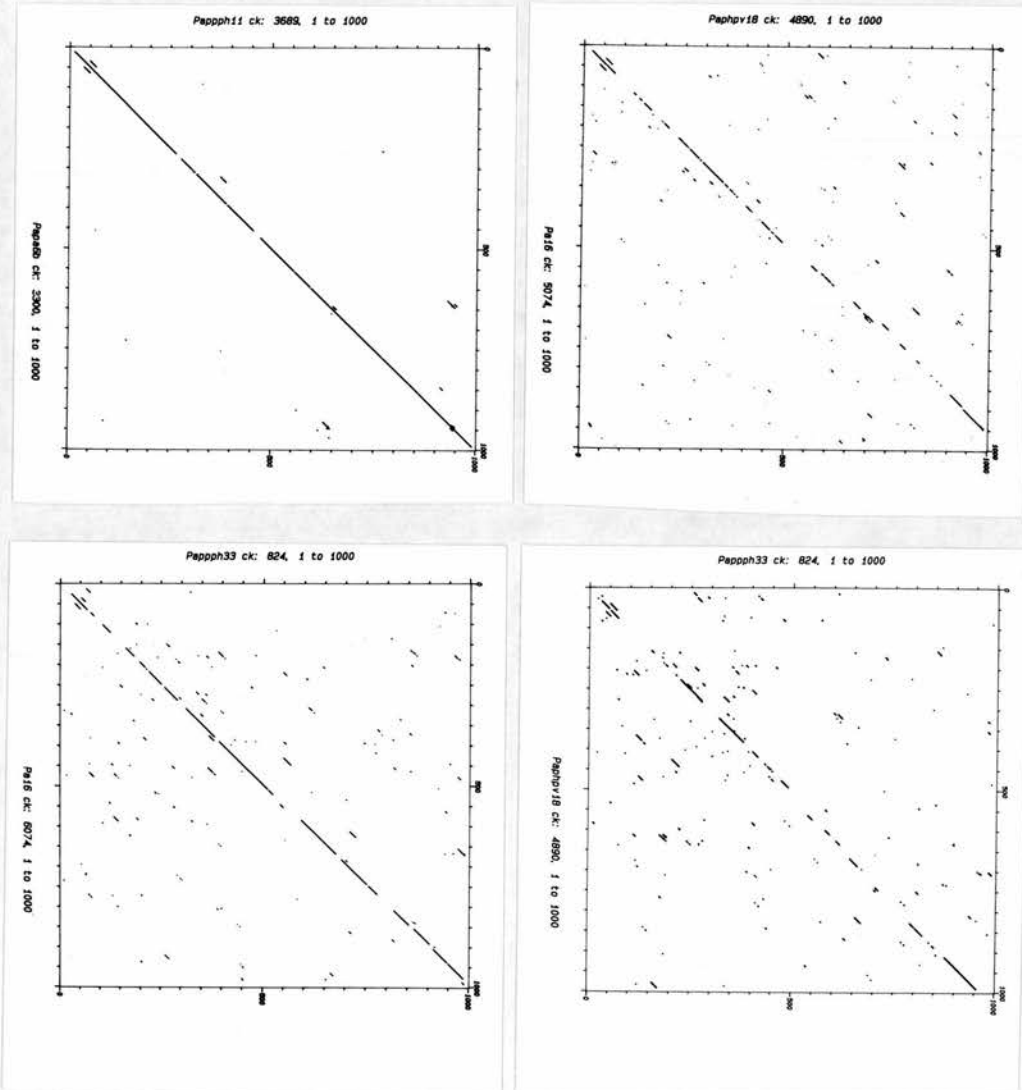


Figure 3.3

Computer generated dotplots of the comparison of homologies of the first 1000 nucleotides (which contain the E6 and E7 sequences) of (1) HPV 6b with HPV 11, using a window of 20 bases in length and a stringency of 15, with each dot plotted representing the occurrence of 15 or more identical bases out of 20 being compared at that position, and of (2) HPV 16 with HPV 18, using a stringency of 13 with a window of 20 bases, (3) HPV 16 with HPV 33, using a stringency of 13 and a window of 20 bases and (4) HPV 18 with HPV 33, using a stringency of 13 and a window of 20 bases. The high density of dots along the diagonals illustrates the strong colinear homologies, and the gaps indicate the local regions of relative nucleotide mismatching, which are appropriate for use as type-specific PCR primers or probe sequences.



regions with a high degree of mismatching were selected - one at either end for primers 1 and 2 and a central sequence for an oligoprobe.

Having found regions of dissimilarity between HPV types, sequences suitable for efficient primers and probes were chosen on the basis of several other criteria, including GC content, predicted secondary structure and 3' complementarity as suggested in guidelines by Saiki et al (1989). An even distribution of GC to AT bases is desirable to allow use of stringent annealing temperatures. Sequences with significant secondary structure are best avoided as this is thought to interfere with primer annealing during PCR. As there are no guidelines to indicate what constitutes "significant" secondary structure, an arbitrary decision was made to consider only sequences where predicted secondary structures known as stem loops had a maximum of 5 base pairings as predicted using UW programs FOLD and SQUIGGLES (figure 3.4). Complementarity between the 3' ends of primer pairs can lead to the formation of PCR artefacts known as "primer dimers" which can reduce the yield of specific amplified product. Previous data have suggested that two terminal complementary bases does not significantly favour primer dimer formation and therefore, primer sequences with a maximum of 2 complementary bases at the 3' end were considered.

The sequences chosen for use as oligonucleotide primer and probes in the detection assay are shown in table 3.5. These sequences had a low enough level of homology with the other HPV types that they would not be expected to bind at temperatures of between 40°C-60°C. To maximise sequence specificity, oligonucleotides had a minimum length of 20 bases which permitted use of high stringency washing conditions for probes

Figure 3.4

Computer generated prediction of the secondary structure of HPV 16 primer 2. This primer is predicted to have 4 base pairs in the stem loop. The 3' end of the primer - which is important for precise initiation of DNA synthesis - is not involved in the base pairing.

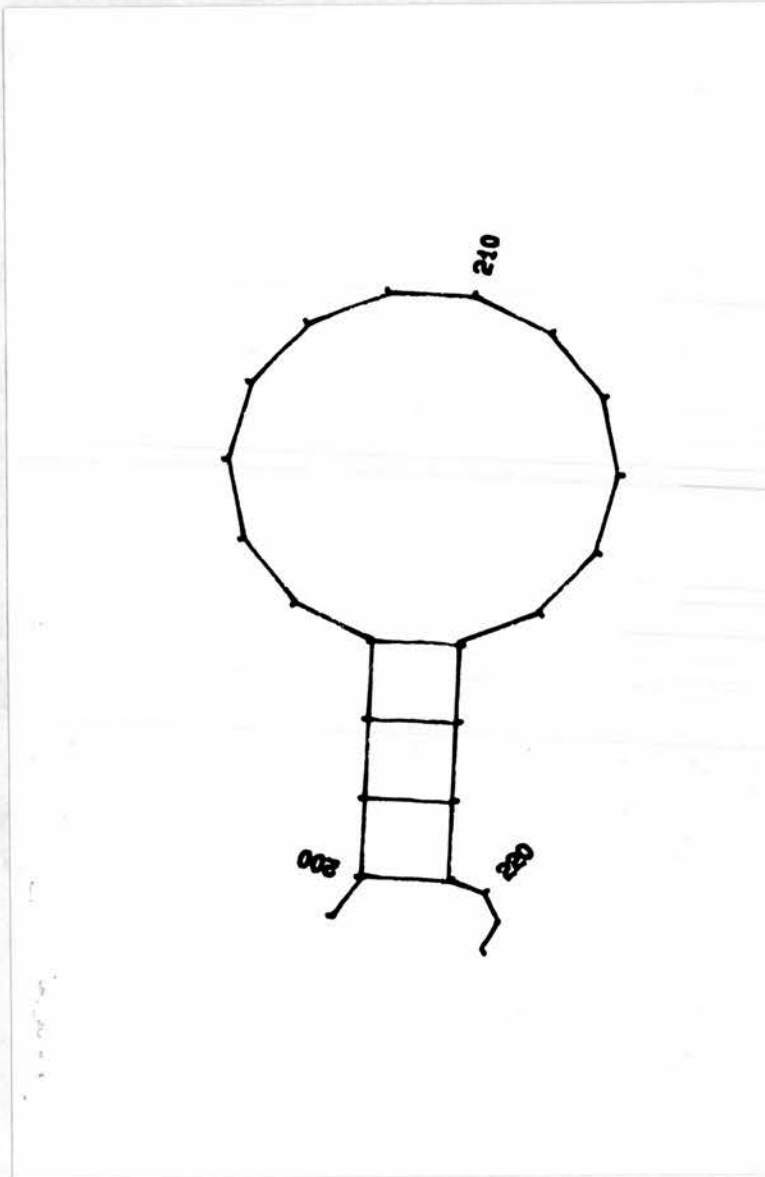


Table 3.5

Nucleotide sequence (5' to 3') of the pairs of PCR primers for each HPV, their positions within the viral genome according to the EMBL database nomenclature and the percentage homology (H) with the equivalent DNA sequence of the most closely matching alternative HPV genome (C).

HPV	PRIMER/ PROBE	SEQUENCE (5' to 3')	POSITION	H	C
6b	Primer 1	CCT GTT TCG AGG CGG CTA TCC ATA	260-283	58	11
6b	Primer 2	GTA CAA TTT AGC TTT ATG AAC CGC GCC TTG GTT	464-496	64	11
6b	Probe	TAA ACA AGA CAT CTT AGA CGT GCT AAT TCG GTG C	380-413	71	11
11	Primer 1	TGT GTG GCG AGA CAA CTT TCC CTT	260-283	58	6b
11	Primer 2	TGG TTA TTT AGT TTT ATG AAG CGT GCC TTT CCC	464-496	64	6b
11	Probe	CAA TGA AGA TAT TTT AAA AGT GTT AAAT TCG TTG T	380-413	71	6b
16	Primer 1	CTG CAA GCA ACA GTT ACT GCG ACG	199-222	42	18
16	Primer 2	CAT ACA TCG ACC GGT CCA CC	495-514	35	18
16	Probe	TAT TAA CTG TCA AAA GCC ACT GTG TCC TGA	412-441	65	18
18	Primer 1	AAA CTA ACT AAC ACT GGG TTA TAC A	378-402	44	16
18	Primer 2	ATG GCA CTG GCC TCT ATA GT	502-521	35	16
18	Probe	CCT GCG GTG CCA GAA ACC GTT GAA TCC AGC	419-448	65	16
33	Primer 1	AAC AGT TAA AAA ACC TTT AAA	378-398	48	16
33	Primer 2	AGT TTC TCT ACG TCG GGA CCT C	528-549	41	16
33	Probe	AAA ACG ACA TGT GGA TTT AAA CAA ACG ATT T	453-483	50	16

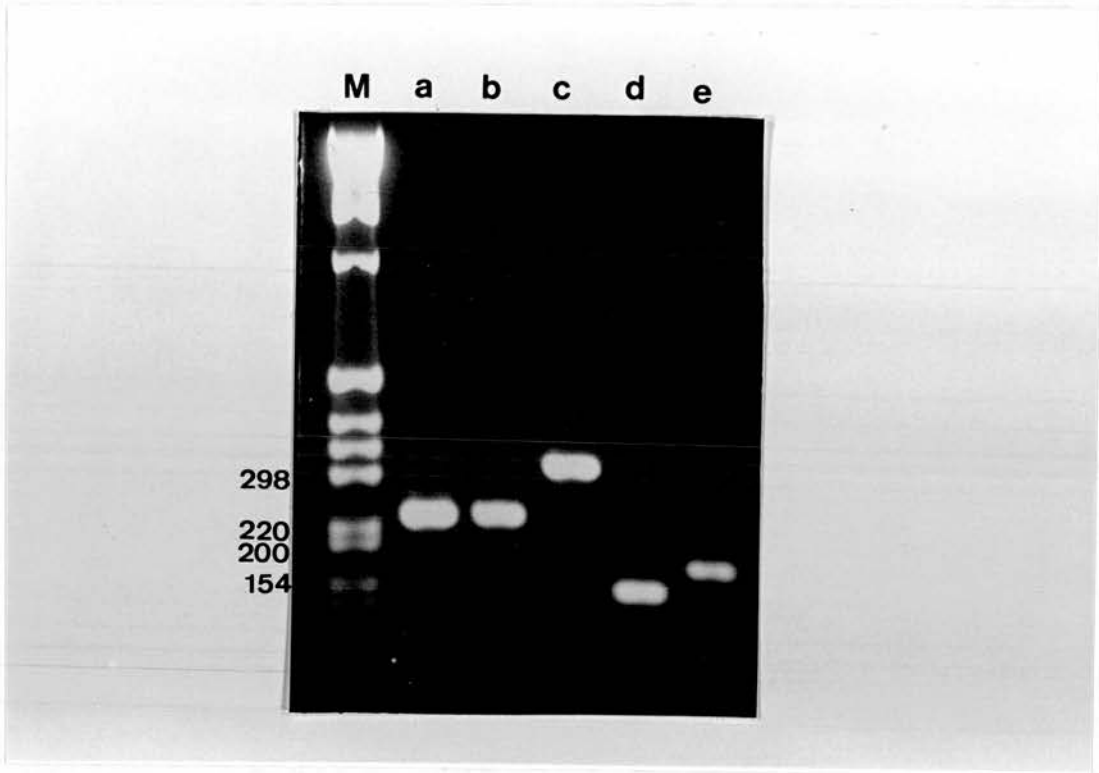
and higher primer annealing temperatures. The sequences of the other HPV types were searched using the UW program FIND to ensure that the selected primers and probes would not cross hybridise. The GC content of the primers chosen varied from 39% to 60% with the exceptions of HPV 18 primer 1 (32%) and HPV 33 primer 1 (19%). Stem loops with equivalent or less than 5 base pairings were predicted for all the primers. No significant complementarity between the 3' ends of paired primers (for each HPV type) was identified, thus limiting the risk of primer dimer formation.

3.2.2 Optimization of conditions for the detection assay

The PCR protocol for amplification of target HPV DNA was varied to find optimum reaction conditions using cloned HPV DNA as template and an initial primer annealing temperature of 42°C. The optimum conditions were considered to be those which gave the highest yield of specific product with the lowest incidence of background judged by subjective examination of agarose gels. For each new batch of enzyme, reactions were set up which had final MgCl₂ concentrations of 1.5mM, 2.5mM and 3.5mM. In every case 1.5mM was found to be optimal. Step times for denaturation, primer annealing and extension of 1min, 2min and 3 min respectively were found to be more efficient than 30 sec, 1 min and 1 min or 1 min, 1 min and 2 min. The primer annealing temperature (Ta) was raised in a series of reactions by 2°C to 60°C. A final Ta of 50°C produced successful amplification using all primer pairs (figure 3.6). This optimization of Ta increases specific binding of primers to target HPV DNA enhancing discrimination against incorrectly annealed primers and reducing misextension of incorrect nucleotides at the 3' end of primers.

Figure 3.6

Agarose gel electrophoresis of amplified DNA using type-specific pairs of primers with cloned DNA for types (a) HPV 6b (fragment length 237bp), (b) HPV 11 (237bp), (c) HPV 16 (316bp), (d) HPV 18 (144bp) and (e) HPV 33 (172bp). A 1kb ladder marker track (M) is included in this and many subsequent gel photographs.



The PCR "plateau" effect was taken into account when deciding upon the number of cycles of PCR to be used in the detection assay. The plateau effect is a term used to describe the attenuation in the exponential rate of product accumulation that occurs during late PCR cycles (Saiki, 1989; Innis and Gelfand, 1990). The point at which any PCR reaction reaches its plateau depends primarily on the number of copies of target DNA originally present in the sample. An important consequence of reaching plateau is that an initially low concentration of non-specific products resulting from mispriming events may continue to amplify preferentially. It is known that HPV copy number can vary considerably in different clinical samples eg. carcinoma derived cell lines SiHa and CaSki contain 1-2 and 400 - 500 copies of HPV 16 respectively (Pater and Pater, 1985). To achieve maximum amplification yet avoid amplifying background products associated with the plateau, the standard 30 cycles as suggested by Saiki (1989) was used. To determine the threshold sensitivity of the assay 35 cycles of PCR were used in order to increase the probability that the plateau was reached for this small number of HPV copies.

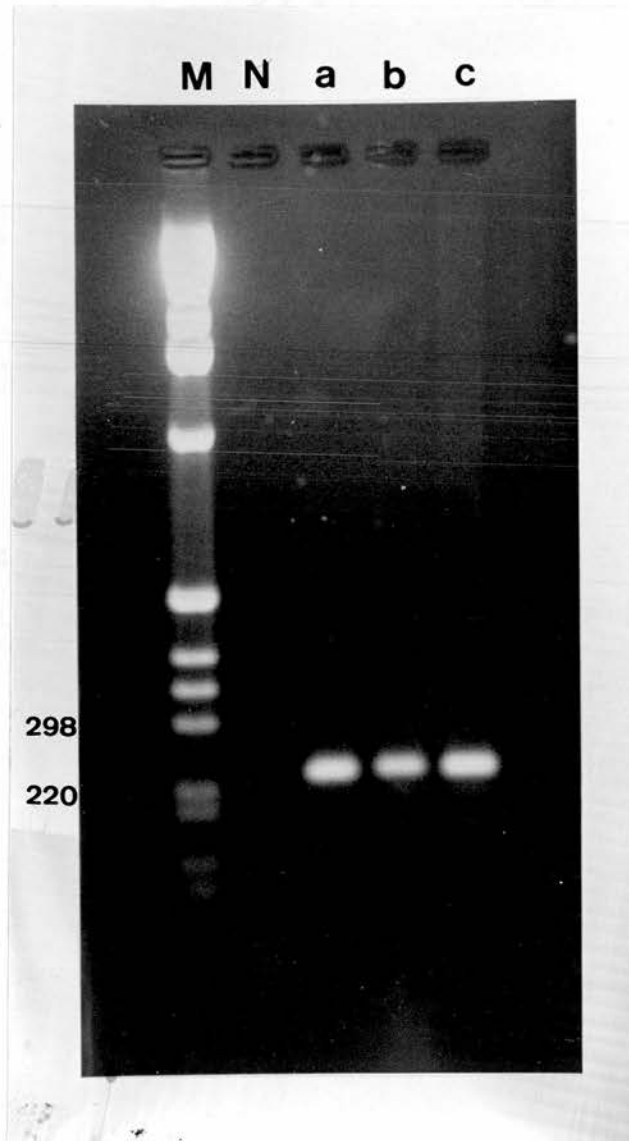
Successful amplification of HPV DNA was achieved using a variety of templates including frozen sections, paraffin wax sections and DNA extracted and purified from both fresh tissue and paraffin wax sections (figure 3.7).

3.2.3 Validation of the detection assay for HPV type specificity

The HPV type specificity of the assay lies in both the primers and the oligoprobes. Specificity of primer directed amplification was assessed for each set of primers with template DNA of the 5 HPV types, 6b, 11, 16, 18 and 33. Amplified DNA fragments were only generated when primer

Figure 3.7

Agarose gel electrophoresis of amplified DNA using HPV 11 primers with samples of a vulvar wart harbouring HPV 11, in the form of extracted DNA (a), frozen sections (b) and paraffin wax embedded sections (c); a 1kb ladder marker track (M) and a negative control track (N) are included.



pairs were used with template DNA of the appropriate type. No amplification occurred when primers of one type were applied to template DNA of another (figure 3.8).

Confirmation that amplified DNA contained the appropriate HPV DNA target sequence was obtained from dot blots hybridised with type specific oligoprobes and from restriction endonuclease mapping using enzymes which generate diagnostic fragments from each of the 5 HPV types (figure 3.9).

To establish type specificity of oligoprobe hybridisation, the amplified target DNA from all 5 HPV types were dot blotted onto nitrocellulose filters and tested for cross-hybridisation using the 5 HPV type-specific oligoprobes. Each oligoprobe generated a positive hybridisation signal only with amplified target DNA of the same HPV type (figure 3.10).

3.2.4 Validation of the detection assay for sensitivity

The sensitivity of the assay was assessed using cultured cervical carcinoma cell lines with a known HPV DNA content. Test samples consisted of serial dilutions of HeLa cells (containing 30-40 copies of HPV 18 per cell) and SiHa cells (containing 1-2 copies of HPV 16 per cell), mixed with 10^4 control Raji lymphoblastoid cells (without any HPV)(Pater and Pater, 1985).

Using appropriate oligonucleotide primers for 35 cycles of the PCR, amplified target DNA sequences were consistently detected by gel electrophoresis from reactions containing 5 HeLa cells (data not shown) or five SiHa cells (figure 3.11A). Quadruplicate reactions, nominally

Figure 3.8

The specificity of HPV 33 primers to direct amplification by PCR of only HPV 33 was tested using cloned HPV genomes as templates. This shows agarose gel electrophoresis of the products of PCRs using HPV 33 primers with template plasmid DNA of types (a) HPV 6b, (b) HPV 11, (c) HPV 16, (d) HPV 18 and (e) HPV 33. A 1kb ladder marker track (M) is included. Positive amplification is achieved only with HPV 33 template. Comparable results were achieved for the other HPV primer pairs.

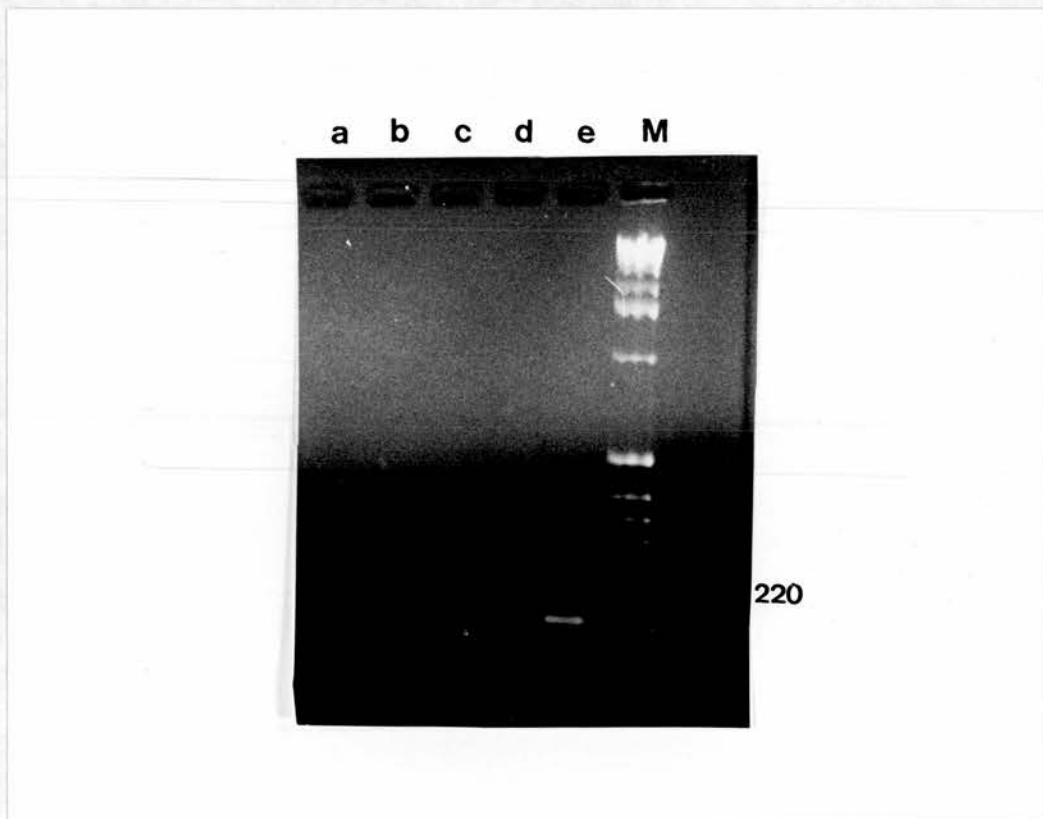


Figure 3.9

Amplified DNA from each of the HPV types was identified by restriction enzyme mapping, with the sizes of digested DNA fragments determined by agarose gel electrophoresis. A unique pattern of DNA fragments was obtained for each HPV type/enzyme combination: (a) HPV6b/DdeI gave 133 and 104bp fragments; (b) HPV 11/DdeI gave 194 and 43 bp fragments; (c) HPV 16/HinfI gave 200, 76 and 40bp fragments; (d) HPV 18/HinfI gave 81 and 63bp fragments; (e) HPV 33/DraI gave 79, 75 and 18 bp fragments. In some reactions small quantities of undigested amplified DNA products are visible. A 1kb ladder marker track is included (M).

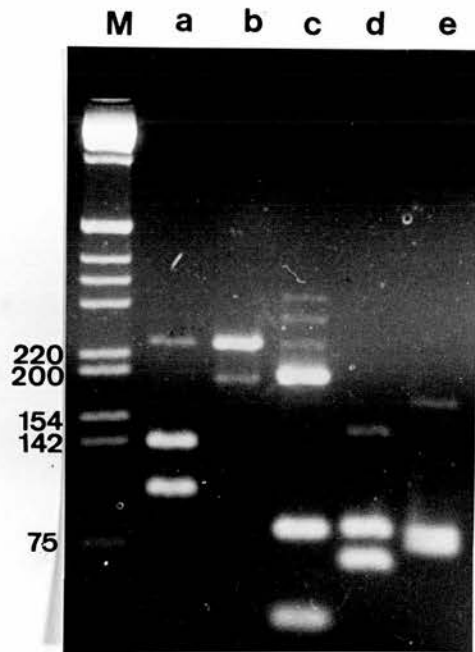


Figure 3.10

Dot blot showing HPV type-specificity of oligoprobe hybridisation to amplified DNA. DNA was immobilised onto the nitrocellulose filter undiluted and in ten- and one hundred-fold dilutions using PCR generated amplified DNA from HPV types 6b, 11, 16 and 18 and acting as negative and positive controls plasmid DNA of pBR322 (neg) and of HPV 11 (pos) respectively. The filter was hybridised with the biotinylated oligoprobe for HPV 11 which was detected by a streptavidin and alkaline phosphatase reaction. The probe identifies only amplified DNA of the same HPV type.

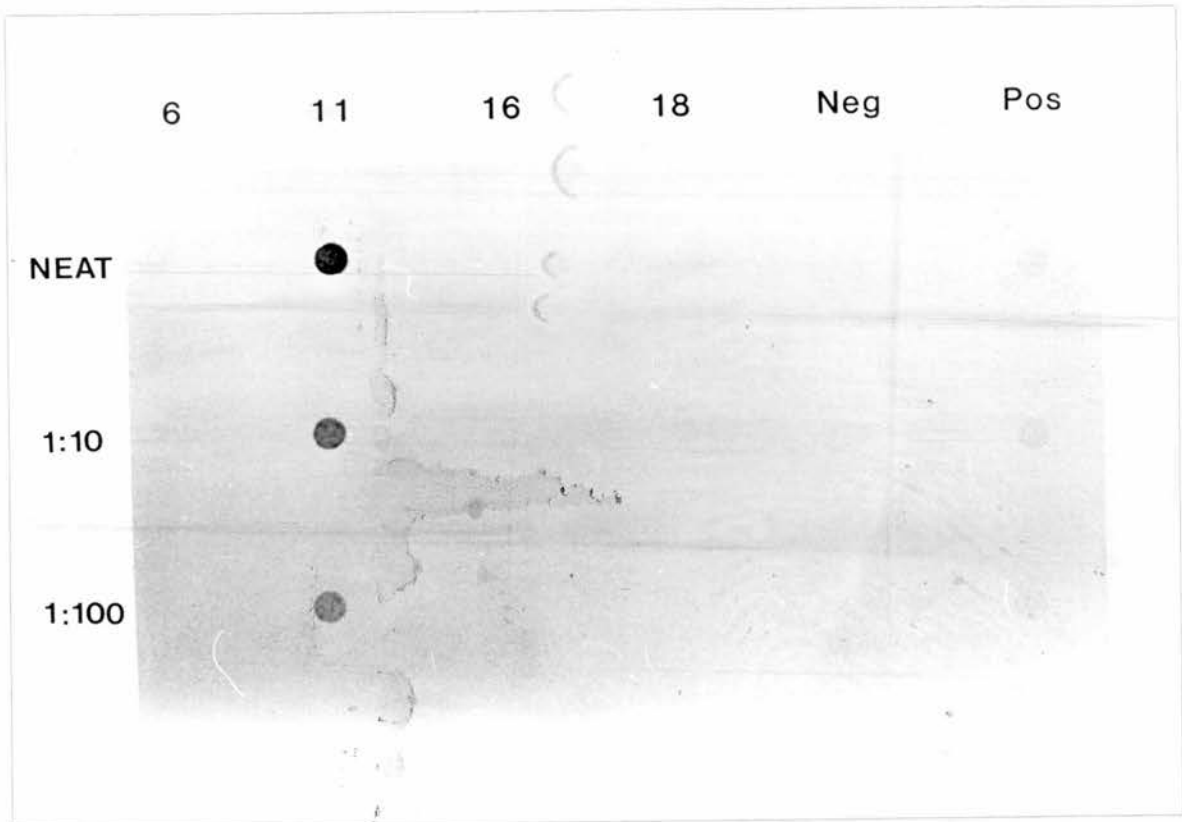
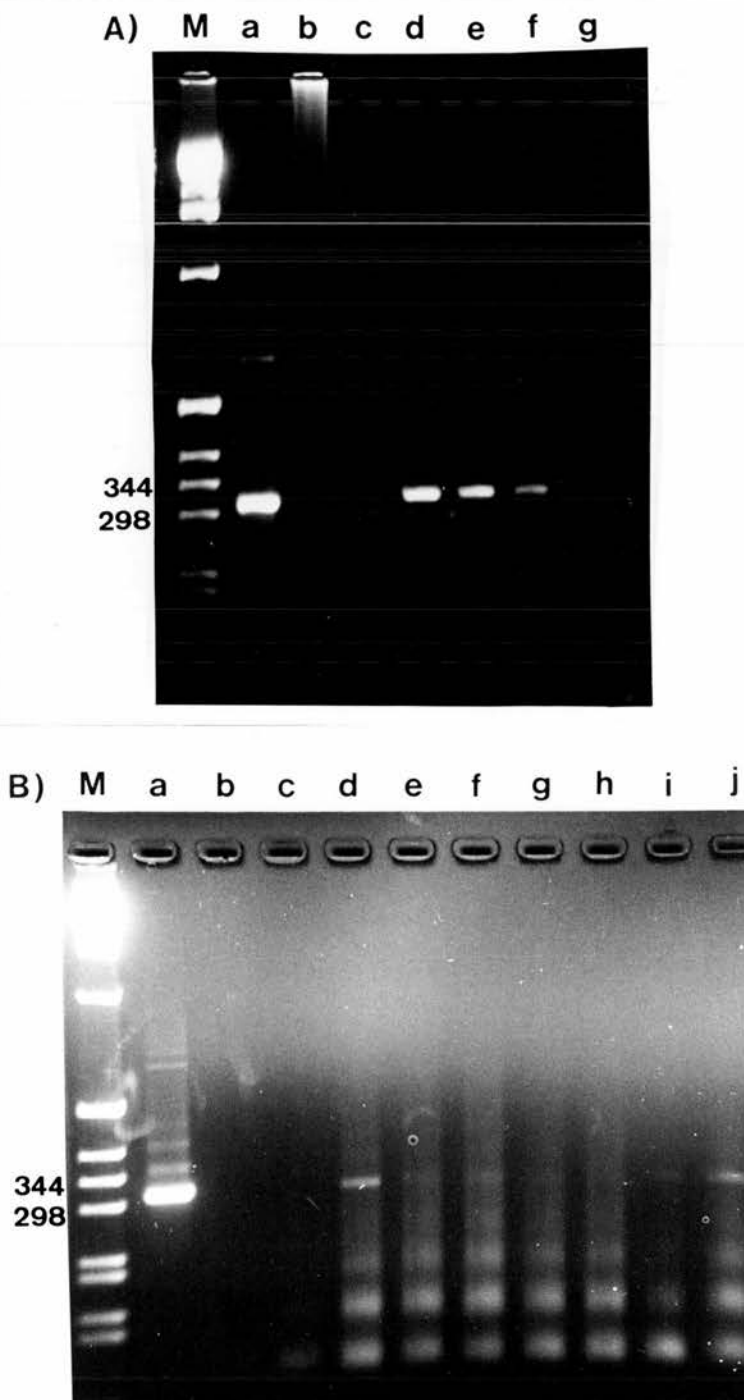


Figure 3.11

Sensitivity of the assay is shown by agarose gel electrophoresis of amplified viral DNA from SiHa cells directed by primers for HPV 16. SiHa cells were serially diluted against a background of 10^4 Raji cells. (A) shows reactions containing; no SiHa cells (c); 10^3 SiHa cells (d); 100 SiHa cells (e); 10 SiHa cells (f) and 5 SiHa cells (g). (B) shows the PCR products from several of the quadruplicate reactions; tracks (c-f) are of reactions nominally containing 3 SiHa cells; tracks (g-j) are of reactions nominally containing 4 SiHa cells. Included in both (A) and (B) are 1kb ladder tracks (M); positive control reactions of cloned HPV 16 plasmid DNA (a); and negative control template-free reactions (b).



containing 4, 3, 2 or one SiHa cell were also analyzed. Two out of four reactions containing three or four cells were positive and all of the reactions containing one or two cells were negative (figure 3.11B). Thus, the results demonstrate a threshold sensitivity of between 3 and 5 cells (3-10 integrated HPV DNA copies).

3.3 Discussion

The assay was successfully applied to material from a variety of sources. When amplified products were electrophoresed on an agarose gel extracted DNA gave the least smearing, followed by frozen sections then paraffin wax embedded sections. An accurate comparison of the efficiency of PCR as applied to material from different sources was not possible as this would have required the use of equivalent amounts of differently processed material from the same case.

The HPV type specificity of detection assay described here was demonstrated by amplification of HPV DNA only where primers were used with template DNA of the appropriate type; the absence of amplification where primers of one type were applied to template DNA of another; confirmation of HPV type achieved by dot blot hybridisation of amplified products with HPV type specific oligoprobes.

This specificity was achieved through design of separate primers and probes with unique sequences for each HPV type. The length of primers allowed use of a stringent annealing temperature reducing the probability of illegitimate primer annealing to near complementary sequences. This assay therefore could be seen to have an advantage over several *in situ* hybridisation methods which cannot differentiate between HPV types 6

and 11 (Schneider et al., 1991) and also some early PCR assays which have been found to have problems arising from the cross amplification of different HPV types (Syrjanen, 1990).

Other PCR based detection studies have used consensus or common primers that bind to the DNA of a broad spectrum of HPV genotypes (Snijders et al., 1990). In such studies specificity may be compromised as low annealing temperatures (40°C) are required to allow the binding of primers to a range of target sites not all of which are a perfect match. Furthermore, it is worth noting that the use of separate type specific primers will give precise data about HPV types of known oncogenic potential whereas general primers will pick up other HPV types of unknown potential.

The use of PCR amplification conferred high sensitivity upon the assay, shown to be capable of detecting between 3-10 copies of HPV 16 in a background of 10,000 sample cells. PCR is known to be more sensitive than *in situ* hybridisation (which requires few cells and 20-800 HPV genomes per cell depending on the assay), FISH (which requires few cells and at least 10^4 HPV genomes per cell), dot blot (which requires 500ng DNA and 1 HPV genome per cell) and Southern blotting (which requires 10ug DNA and 0.1 HPV genome per cell) in the detection of HPV DNA (Syrjanen, 1990; Morris et al., 1990; Tham et al., 1991; Arends, 1991).

Also, by siting primers for the PCR assay within the E6 gene both episomal and integrated HPV can be detected whereas, other techniques such as *in situ* hybridisation may require a high copy number per cell

such as occurs in foci of replication of episomal viral DNA and may fail to detect integrated HPV DNA which can be present at low copy number (eg. SiHa contains 1-2 HPV copies/cell).

The use of a highly sensitive PCR assay for the detection of HPV DNA requires the adoption of stringent precautions to avoid and detect contamination by even trace quantities of HPV DNA from other sources (Tidy and Farrell, 1989). These sources may include previously processed pathological specimens, carryover of amplified DNA products or aerosol formed from solutions of plasmids containing cloned HPV DNA (Kwok et al, 1990). In order to reduce the incidence of false positives anticontamination primers have been incorporated into some assays (Van den Brule et al., 1989). These primers spanned the HPV cloning site and thus avoided detection of HPV plasmid DNA contamination. However contamination from other sources would not be detected using this approach. Furthermore, the siting of anticontamination primers within the L1 or E1 genes (cloning sites of HPV 16 and HPV 18 respectively), which are often deleted or disrupted following integration, could lead to false negatives if used alone.

The myocardial tissue and template-free negative controls used in this assay should detect contamination from plasmid DNA, amplified products and previously processed material occurring either during the preparation of tissue for PCR, or the setting up of PCR. The precautions taken to avoid contamination appear to have been adequate as no false positives were detected since their adoption in over 500 PCR analyses.

The ability to detect trace amounts of DNA in clinical samples raises the

question of their biological significance. The presence of trace amounts of HPV DNA in non-neoplastic epithelium may be due to latent HPV infection occurring, with little significant viral replication. Trace amounts of HPV DNA found in CIN lesions or cervical carcinomas may contribute to the neoplastic phenotype. Support for this possibility comes from the fact that SiHa cells - derived from a cervical carcinoma- contain only 1 to 2 copies of integrated HPV DNA of which E6 and E7 genes are expressed (Pater and Pater, 1988). Thus it is possible that a single high risk genome could contribute to a neoplastic phenotype. This being the case its detection would require a highly sensitive assay .

CHAPTER 4

DETECTION OF HPV IN CERVICAL NEOPLASIA

4.1 Introduction

HPV is frequently found in carcinoma of the cervix and its precursor CIN, supporting a role for the virus in cervical carcinogenesis. However the precise involvement of HPV is not fully understood. Studies have shown that HPV in co-operation with other factors has the potential to fully transform human keratinocytes but it is not known where in the CIN-cancer sequence HPV acts. Furthermore, while the levels of risk assigned to HPV 6/11 and HPV 16/18 are widely accepted, there are conflicting reports of an association between HPV 18 and glandular cervical carcinoma and a more rapidly progressive type of carcinoma (Kurman et al., 1988; Wilczynski et al., 1988; Griffin et al., 1991; Ji et al., 1991).

In this study, the highly sensitive and specific PCR based assay was applied to cases covering the spectrum of cervical disease including normal tissue, CIN, squamous and glandular carcinomas in order to define more clearly their levels of association with specific HPV types. In particular, this study aims to gain an understanding of the relationship between HPV type and neoplastic aggression, between HPV type and tumour differentiation and finally the stage or stages in the CIN-cancer sequence at which HPV acts.

4.2 Cases Studied

The PCR assay as described in chapter 3 was applied to twenty four control cases (mean age 35 , SD 11.29, range 17-56) , twenty cases each of CIN I (mean age 30.3, SD 8.38, range 19-49), CIN 2 (mean age 28.65, SD 7.43, range 19-45) and CIN 3 (mean age 31.4, SD 8.43, range 22-50), as well as 26 cases of squamous cell carcinoma (mean age 36.6, SD 11.59, range 20-58) 16 cases of adenocarcinoma (mean age 39.7, SD

7.48, range 28-58) and 4 cases of adenosquamous carcinoma (mean age 33.2, SD 5.85, range 26-40).

4.3 Results

All 24 normal control samples which consisted of frozen cervical tissue taken at forensic autopsy were negative for HPV DNA. HPV DNA was detected in 25% of CIN I cases, 60% CIN 2 cases, 60% CIN 3 cases and in 80% of carcinomas. HPV DNA was detected in 75% of adenocarcinomas, 81% of squamous carcinomas and all 4 adenosquamous carcinomas. The prevalence of individual HPV types in these lesions reveals HPV 16 to be the most common in all grades of lesion accounting for 60%, 75%, 92% and 62% of the total HPV positive cases in CIN 1, CIN 2, CIN 3 and all carcinomas respectively (figure 4.1). No HPV 6b or 33 was identified in any of the cases examined. HPV 11 was found in only one lesion which was diagnosed as CIN 1. HPV 18 was found in 6 out of 26 (23%) cases of squamous carcinomas, 1 of 4 (25%) adenosquamous carcinomas and 7 of 16 (44%) glandular carcinomas cases (figure 4.2). Two cases of adenocarcinoma contained both HPV 16 and 18.

All cases found to be negative for the 5 HPV types by PCR were subjected to amplification of a reference gene which produced amplified DNA products of the appropriate size (figure 4.3), demonstrating that this DNA was intact and that there was no inhibition of Taq polymerase in these reactions. Amplified DNA taken from cases found to be HPV positive from the PCR assay was subjected to dot blot hybridisation with HPV type specific oligoprobes for confirmation of HPV type (figure 4.4).

Figure 4.1

Agarose gel electrophoresis of amplified DNA from cases of CIN using frozen sections as template and primers for HPV 16 DNA. Eight cases are positive (b-f, h, i, l) and three cases are negative (g, j, k) for HPV 16. Included are a template-free negative control track (a) and 1kb ladder track (M).

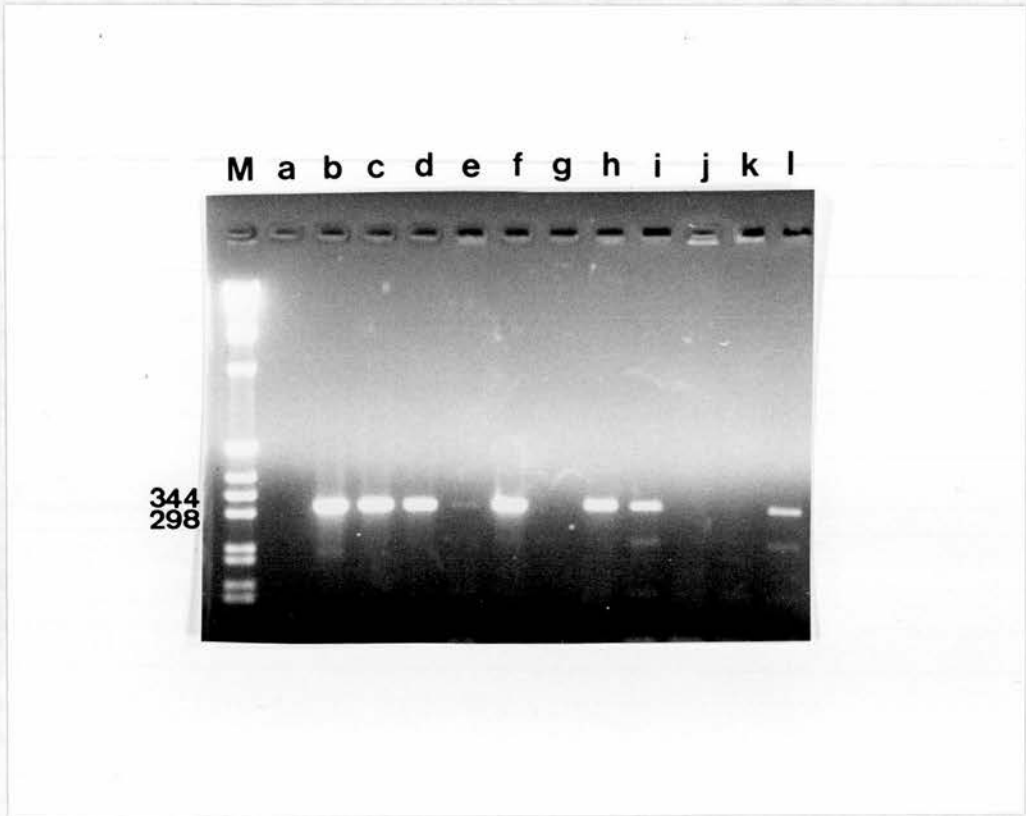


Figure 4.2

Agarose gel electrophoresis of amplified DNA from cases of adenocarcinoma using extracted DNA from paraffin wax-embedded tissue as template and primers for HPV 18 DNA (c-l). Five cases are positive and five cases are negative for HPV 18 DNA. Included are a cloned HPV plasmid positive control track (a), a template free negative control track (b) and a 1kb ladder marker track (M).

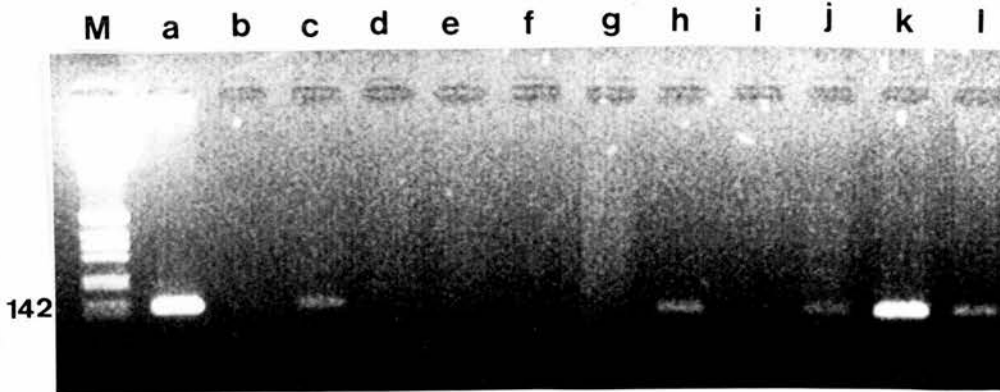


Figure 4.3

The presence of amplifiable DNA in clinical material was shown by PCR amplification of a reference gene c-Ha-ras 1. Agarose gel electrophoresis shows amplified DNA from nine normal control cases (c-k); a template free negative control track (a); a cloned HPV plasmid DNA positive control track (b) and 1kb ladder marker track (M).

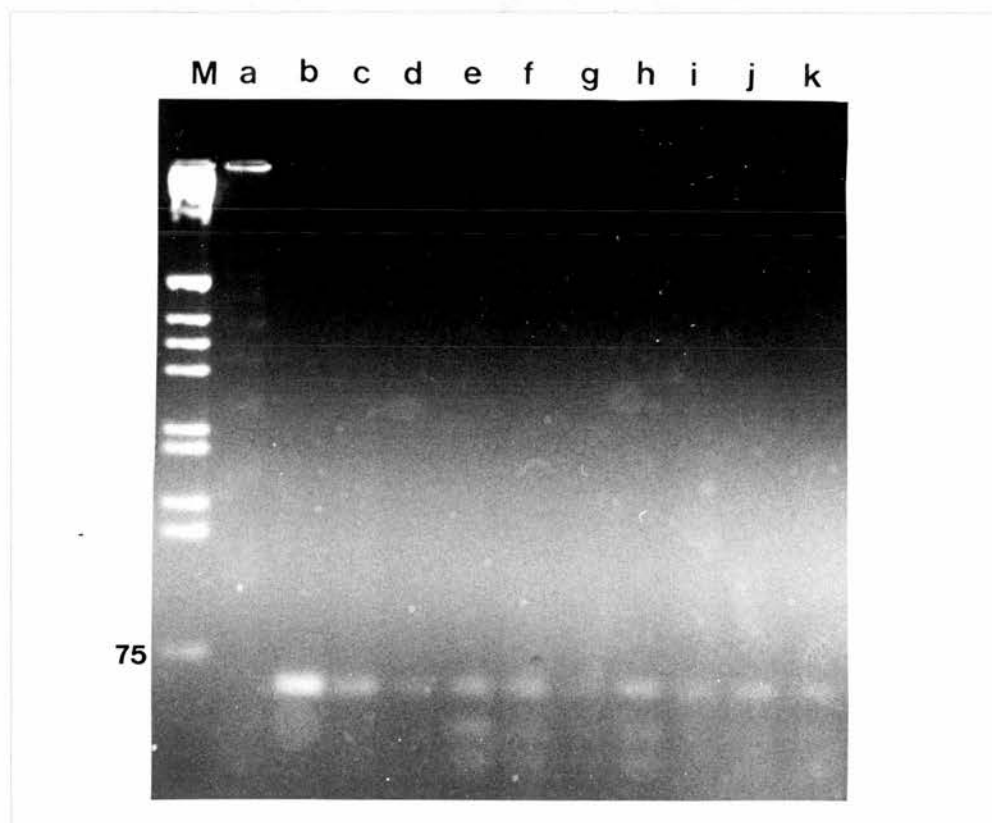
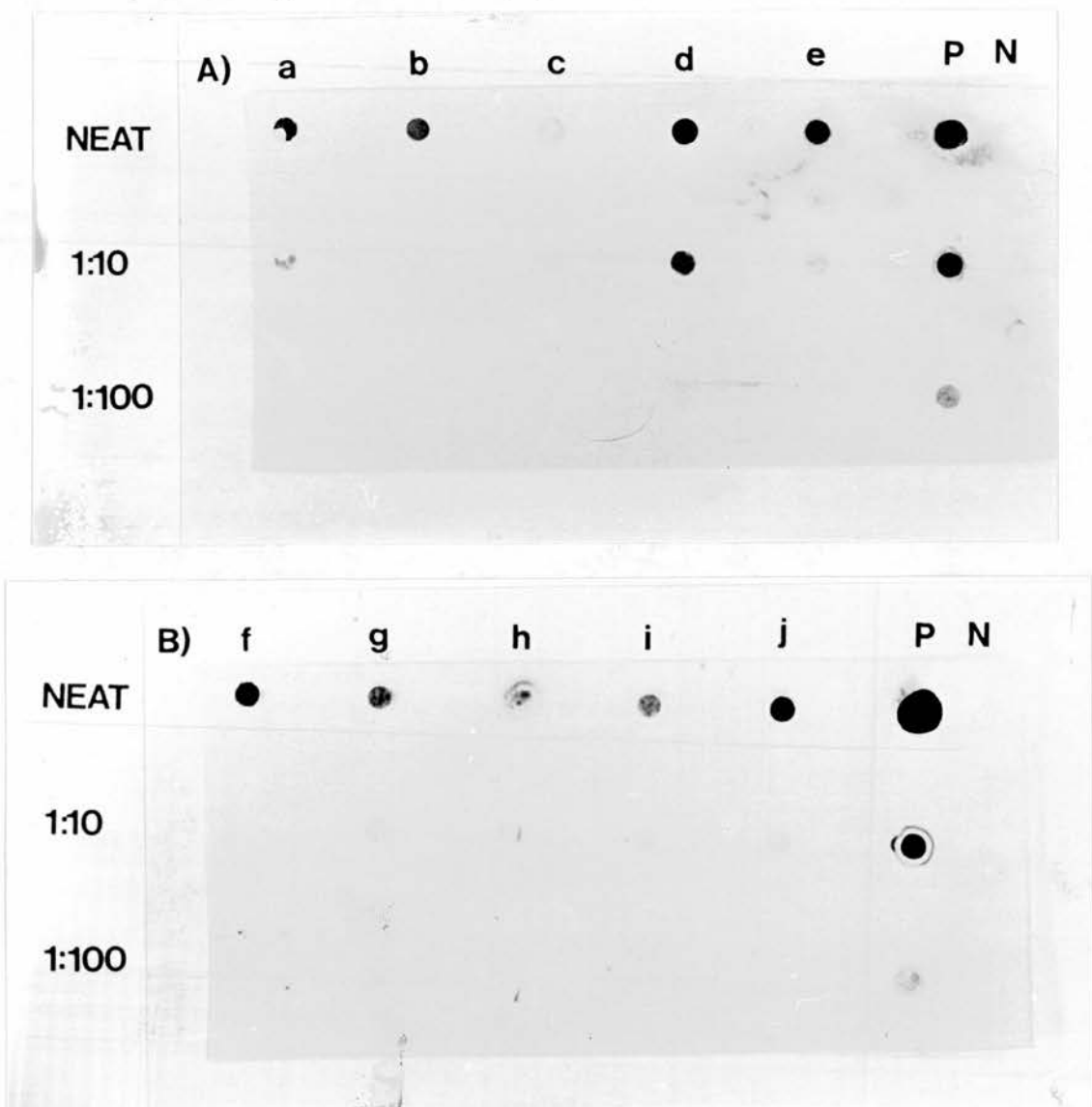


Figure 4.4

Confirmation of HPV type by oligoprobe hybridisation of PCR products. (A) shows the PCR products from three cases of adenocarcinoma (a-c) and two cases of squamous carcinoma (d, e) undiluted, and diluted ten- and one hundred-fold detected with a biotinylated HPV 16 oligoprobe. (B) shows PCR products from 4 cases of adenocarcinoma (f, g, i, j) and one case of adenosquamous carcinoma (h) undiluted and diluted ten- and one hundred-fold, detected with a biotinylated HPV 18 oligoprobe. Both (A) and (B) include a positive control of amplified cloned HPV plasmid DNA undiluted and diluted ten- and one hundred-fold (P) and a negative control of plasmid pBR322 DNA (N).



The results for HPV prevalence in adenocarcinomas were adjusted to exclude cases which contained adjacent CIN (table 4.5). Eleven of the sixteen cases examined contained no adjacent CIN. Eight of these eleven cases (73%) contained HPV DNA, five (45%) contained HPV 18 and four (36%) contained HPV 16 (one case contained both HPV 16 and 18). There was no significant difference in the mean ages or proportion of cases over 35 years of age between HPV containing cases and those found to contain no HPV DNA of types 6, 11, 16, 18 or 33 (X^2 test). To compare the effects of HPV 16 and HPV 18 on neoplastic progression various CANCER/CIN prevalence ratios were calculated (table 4.6). These show a 1.9 - 2.8 fold difference between virus types.

The HPV prevalence data from 130 samples of cervical tissue are summarised in table 4.7. The statistical significance (X^2) of comparative HPV prevalence in normal tissue, CIN 1, CIN 2/3, squamous carcinoma and adenocarcinoma is summarised in table 4.8.

4.4 Discussion

No HPV DNA was found in any of the 24 control cervixes from forensic autopsy. These negative results contrast with HPV prevalence figures of between 0 and 70% from studies using PCR based methods (Young et al., 1989; Manos et al., 1990) and between 9 and 19.5 % using non-PCR based methods (deVilliers et al., 1987; Wickenden et al., 1987). These widely differing rates may be attributable to several factors including, the varying sensitivity of each particular detection assay, the source of the control population, or the different cervical sampling methods used. The different sensitivities of the commonly used detection methods have been widely reported (Munoz et al., 1988; Melchers et al., 1989; Tham et

Table 4.5

Overall prevalence of HPV DNA in glandular carcinomas with adjacent CIN and those without.

	HPV +	HPV -	TOTAL
CIN +	4	1	5
CIN -	8 (73%)	3 (27%)	11
TOTAL	12 (75%)	4 (25%)	16

Table 4.6

CANCER/CIN prevalence ratios for HPV 16 and 18.

Prevalence Ratio	HPV 16	HPV 18	18/16 Ratio
All Cancer: All CIN	1.42	3.75	2.6
Squamous cancer: All CIN	1.5	2.87	1.9
All Cancer: CIN 2/3	1.08	3	2.8
Squamous Cancer: CIN 2/3	1.1	2.3	2.1

Table 4.7

The frequency of HPV 6b, 11, 16, 18 and 33 DNA in normal tissue and cases of CIN 1, CIN 2, CIN 3, squamous carcinoma, adenocarcinoma and adenosquamous carcinoma by PCR.

DIAGNOSIS	N	HPV POSITIVITY (%)					TOTAL HPV POSITIVE (%)
		6	11	16	18	33	
CONTROLS	24	-	-	-	-	-	-
CIN 1	20	-	1 (5)	3 (15)	1 (5)	-	5 (25)
CIN 2	20	-	-	9 (45)	3 (15)	-	12 (60)
CIN 3	20	-	-	11 (55)	1 (5)	-	12 (60)
SqCa	26	-	-	15 (58)	6 (23)	-	21 (81)
AdCa	16	-	-	7 (44)	7 (44)	-	12 (75) ^a
AdSqCa	4	-	-	3 (75)	1 (25)	-	4 (100)

a. 2 cases positive for both HPV 16 and 18.

Table 4.8

P values of the X^2 statistic (DF=1) for the comparison of overall HPV prevalence in normal tissue, CIN 1, CIN 2/3, squamous carcinoma and adenocarcinoma.

	CIN 1	CIN 2/3	Sq Ca	Ad Ca
Controls	<0.001	<0.001	<0.001	<0.001
CIN 1		<0.001	<0.001	<0.001
CIN 2/3			<0.1	<0.01
Sq Ca				<0.5

al., 1991). While the insensitive FISH and dot blot techniques may underestimate HPV prevalence, the contamination often associated with PCR based methods may result in false positives and an overestimate of HPV prevalence. The method used here was validated for sensitivity and incorporated negative controls designed to detect contamination.

The source of the control population examined in many studies has included patients attending sexually transmitted disease clinics, patients successfully treated for CIN and patients attending colposcopy clinics, arguably unrepresentative of the normal population. In support of this, a recent study compared HPV prevalence in two different groups of women; a symptom free population involved in a district call scheme for cervical cancer and gynecological outpatients population containing women visiting the clinic for a wide range of gynecological complaints or for control of hormonal contraception. HPV prevalence in the latter group ranged from 9.2% to 21.5% depending on whether patients had a history of cervical pathology. This was significantly higher than the figure of 3.5% for HPV prevalence in the symptom-free group (van den Brule et al., 1991). The cervical tissue used in the present study came from young women undergoing forensic autopsy following accidental death. This is arguably an apparently random sample more representative of the normal population.

It has been suggested that the different cervical sampling methods used in HPV prevalence studies may account for the varying results (Munoz et al., 1988). Commonly used methods include the cervical scrape and lavage which sample a large area of exocervical epithelium consisting largely of superficial cells. Here, histologically normal full thickness

epithelium was taken from the cervical transformation zone.

The comparison of HPV prevalence in CIN lesions found in different studies proves difficult because of the different detection methods used and the manner in which results are presented (that is figures for HPV 6 and 11 or CIN 1 and CIN 2 are often combined). The finding of HPV in 60% of CIN 2 lesions is higher than previously reported for CIN 2 biopsies as analyzed by dot blot whereas the finding of HPV in 60% of CIN 3 is lower than reported for CIN 3 biopsies in other studies (Pater et al., 1986). The prevalence of HPV in CIN 1 and CIN 3 biopsies in this study is not as high as in other studies using scrapes (Melchers et al., 1989). These differences may relate to the focussed sampling of punch biopsies on the abnormal region, compared with the widespread sampling of scrapes, which may include distinct regions of viral replication not necessarily relevant to a particular CIN lesion.

The figure of 81% for HPV prevalence in squamous carcinoma is in broad agreement with the findings of several other groups using PCR detection assays, whereas the finding of HPV types 16 and 18 in 75% of the 16 adenocarcinomas used in this study is considerably higher than reported in some recent studies (Griffin et al., 1991, Young et al., 1991). One of these studies found no HPV DNA of types 6, 11, 16 or 18 in 21 cases of adenocarcinoma (Young et al., 1991). One explanation for this low HPV prevalence may be the detection method used - *in situ* hybridisation (ISH) - which is of very low sensitivity compared with PCR (Levi et al., 1989). In support of this, a study using both ISH and PCR to detect HPV DNA in cervical adenocarcinomas found only one of five cases giving a positive PCR result also gave a positive ISH signal

(Griffin et al., 1991). Griffin et al detected HPV types 16 and 18 in only 31% of glandular cancers. While this assay was carefully validated for both specificity and sensitivity, several alternative explanations were offered by the authors for the low frequency of HPV detected as compared with previous studies. These included possible geographical differences in HPV carriage given the study in question came from the UK whereas many of the previous studies originate from the US or Australia; the existence of one population of adenocarcinoma occurring in young women (under 35 years of age) which is related to HPV infection and another in older women which is not associated with a sexually transmitted agent; contamination by adjacent CIN (a possibility also suggested by Young et al (1991) to explain the low detection rate in their study).

Contamination by adjacent CIN and the possibility of age distribution can be excluded as explanations for the high prevalence of HPV in adenocarcinomas presented here. Both the high sensitivity of the assay and the check for amplifiable DNA in all PCR negative cases, minimised the possibility of false negatives.

It is possible that the low prevalence of HPV detected by Griffin et al (1991) in paraffin wax embedded adenocarcinomas may be due to a lack of DNA amplifiable by PCR. The importance of a check for amplifiable DNA should not be underestimated, especially in view of recent studies which highlight the possible occurrence of false negatives when using PCR on paraffin wax derived tissue. In one study it was found that the amount of PCR product varied inversely with the number of tissue sections used. The authors' attempts to remove this "PCR inhibition" by

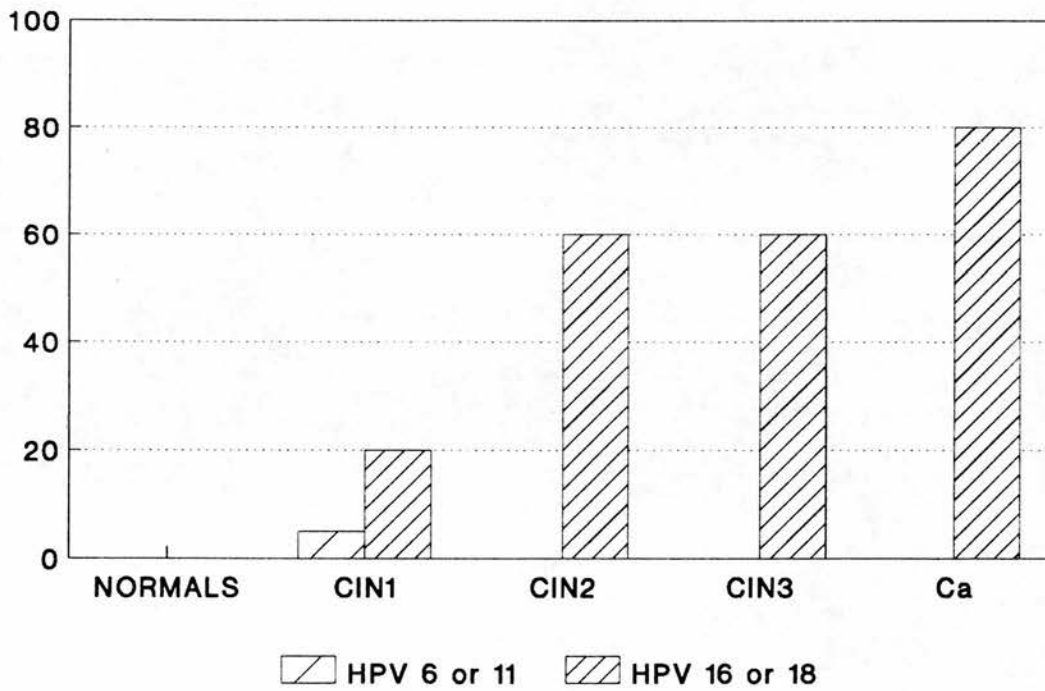
various treatments which included extensive deparaffinisation and proteinase K digestion were unsuccessful (Lo et al., 1989). It has also been found that amplification of extracted digested samples which have been stored is not as successful as is amplification of samples that have been used immediately after digestion (Wright and Manos, 1990).

Prevalence of individual HPV types found in the present study supports the findings of previous studies in that HPV 6/11 are confined to low grade CIN lesions whereas HPV types 16 and 18 are seen with increasing frequency through the spectrum of cervical neoplasia (figure 4.9) supporting a role for these types in the development of cervical carcinoma (Lorincz et al., 1987; Kurman et al., 1988). However, the results of this study emphasise the biological similarity between CIN 2 and CIN 3 and their divergence from CIN 1 as regards HPV prevalence. This finding suggests that CIN 1 lesions may be induced by factors other than HPV infection and thus only CIN 2/3 are the true precursors to invasive cancer. In the CIN-cancer sequence HPV 16 and 18 could be seen as effecting transition from normal tissue or CIN 1 to the CIN 2/3 state.

It seems likely that the presence of HPV 16 or 18 in CIN lesions may indicate a greater tendency for progression and thus, data on HPV may prove to be of use in an improved diagnostic and prognostic system for classification of CIN, possibly leading to the more regular screening or earlier treatment of women where these virus types are detected. The decision whether or not to treat CIN 1 lesions, many of which have been found to regress, appears to be a difficult one (Syrjanen et al., 1987). Prospective clinical and viral studies aimed at delineating long term

Figure 4.9

Bar chart of the prevalence of HPV 11 and HPV 16/18 in 24 controls and 20 cases each of CIN 1, CIN 2 and CIN 3 and 46 cases of carcinoma.



behaviour of low and moderate grade CIN lesions based on HPV type may help to establish this as a valuable prognostic indicator.

It has been suggested that there are differences in behaviour of tumours associated with HPV types 16 and 18. HPV 18 has been reported as being more prevalent in glandular carcinoma of the cervix and having a greater tendency toward rapid progression to cervical cancer.

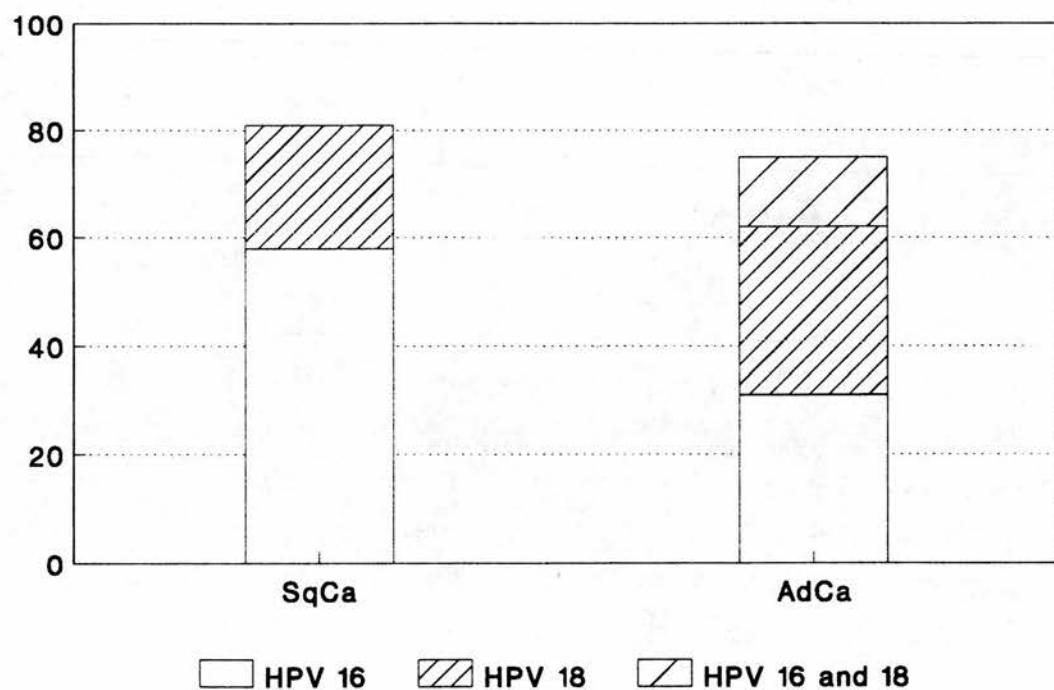
Interestingly, the results of this study show significantly more HPV 16 in squamous carcinomas (58%) than HPV 18 (23%)($P < 0.001$; χ^2 test) but identical frequency in glandular cancers (44%)(figure 4.10). Similar results were reported by Tase et al (1988) using ISH, HPV 18 was found in 40% of adenocarcinomas and in none of the squamous carcinomas whereas HPV 16 was found in 54% of squamous carcinomas and in one of forty adenocarcinomas; and also Wilczynski et al (1988) using Southern blotting, found 50% HPV 16 and 7% HPV 18 in squamous carcinomas and 13% HPV 16 and 50% HPV 18 in adenocarcinomas.

Cervical squamous carcinomas are more common and have a better prognosis than adenocarcinomas (Buckley and Fox, 1989). The association of squamous carcinoma with more HPV 16 than HPV 18 compared with equivalent frequency of the two types in adenocarcinoma suggests that HPV 16 confers a better prognosis in terms of cancer cell differentiation, than HPV 18.

To determine whether the presence of HPV 18 could be associated with mucin production by associated tumours, (an indicator of glandular differentiation) AB/PAS stains were carried out on all cases of

Figure 4.10

Bar chart comparing the relative prevalence of HPV 16 and HPV 18 in glandular and squamous carcinomas.



carcinoma. No correlation was found.

While the reasons for the differences in prevalence of HPV 16 and 18 in cervical adenocarcinomas and squamous carcinomas requires further investigation, two models are postulated. Different HPV types may target different cell populations already committed to either glandular or squamous differentiation. Alternatively, HPV infection may affect epithelial cell differentiation such that HPV 18 tends to induce glandular differentiation, whereas HPV 16 may tend to induce squamous differentiation.

The high prevalence of HPV 16 and 18 in glandular carcinomas may indicate a role for particular viral types in neoplastic progression and it is possible that data on HPV type infection in smears may be useful in detecting the presence of glandular atypia or adenocarcinoma in situ (AIS) since the sensitivity of the Pap smear in detecting preinvasive glandular neoplasms of the cervix appears to be suboptimal.

To investigate the possibility that HPV 18 may have a greater tendency toward rapid neoplastic progression than HPV 16, four CANCER/CIN ratios were calculated for each HPV type. While CIN is believed to be a precursor of squamous carcinoma it is unclear whether adenocarcinoma can also develop from CIN or whether glandular atypia or AIS can progress to squamous carcinoma. For this reason ratios were calculated both from data for all carcinomas and for squamous carcinomas alone. Ratios were calculated from data for all CIN combined and also for CIN 2/3 (which may represent the true precursors of cervical cancer). The amalgamation of HPV prevalence figures for CIN lesions can be justified

as stages one to three are arbitrary divisions in a morphological continuum. The ratios range from 2.3-3.75 for HPV 18 and from 1.08-1.5 for HPV 16 representing a 1.9-2.8 fold difference between virus types suggesting a role for HPV 18 in either a greater risk of progression or a more rapid transition to malignancy. Similarly, Kurman et al (1988) reported a CANCER/CIN ratio of 7.3 for HPV 18 and 1.1 for HPV 16 (a 6.6 fold difference). Thus, from both the comparison of HPV types associated with cancer cell differentiation and CANCER/CIN prevalence ratios it would appear that HPV 18 is more aggressive than HPV 16.

CHAPTER 5

DETECTION OF LARGE SCALE SEQUENCE ALTERATIONS IN THE HPV URR

5.1 Introduction

The results of this and many other studies support a role for HPV in cervical neoplasia. However, there are thought to be other events or factors required for progression to invasive carcinoma. Integration of viral DNA into the host genome may produce an altered pattern of viral gene expression contributing to the neoplastic process. HPV variants with deletion or duplication of URR sequences may have a similar effect.

The common HPV types have been shown to have different oncogenic potentials and these are reflected in their immortalization properties. HPV 18 has been associated with a more aggressive or rapidly progressive type of cervical carcinoma. Interestingly, recent studies have found that the 10 to 50 fold greater immortalization efficiency of HPV 18 as compared to HPV 16 maps to the upstream regulatory region (URR) indicating a possibility that differences in URR sequence may in part account for the different oncogenic potentials associated with particular HPV types (Romanczuk et al., 1991; Villa and Schlegel, 1991). The URR differs between different HPV types while retaining its regulatory functions for viral DNA replication and gene expression (Chong et al., 1990). It is conceivable therefore that different URR sequences will give rise to subtly different patterns of gene expression some of which may affect the neoplastic process.

The results of several studies have indicated a high degree of URR sequence divergence within HPV types (Deau et al., 1991; Chan et al., 1992). HPV variants with alterations to the URR have been discovered in a variety of genital cancers and unusual lesions (table 5.1, figure 5.2). It has been suggested that pathogenicity of these HPV variants may be

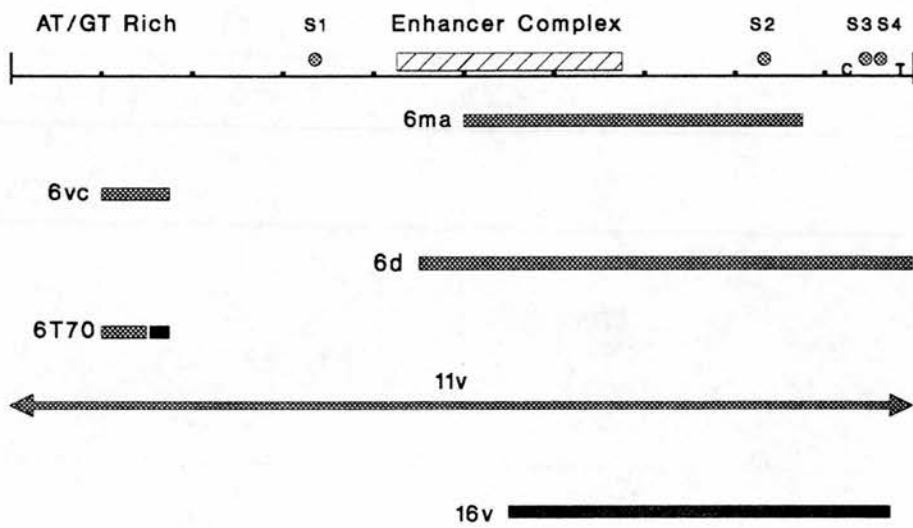
Table 5.1

Several HPV variants are shown including, the major duplications (+) or deletions (-) and their position in the HPV genome. The lesions from which these subtypes were first isolated and appropriate references are also shown.

VARIANT	+/-	POSITION OF +/- SEQ	LESION	REFERENCE
6ma	+	7681 - 7836	conyloma of nipple	Kulke et al., 1989
6vc	+	7302 - 7363	vulvar carcinoma	Rando et al., 1987
6d	+	7528 - 84	Buschke-Lowenstein tumour	Boshart & zur Hausen, 1986
6-T70	+	7300 - 7323	vulvar carcinoma	Kasher & Roman, 1988
6-T70	+	7303 - 7356	vulvar carcinoma	Kasher & Roman, 1988
6-T70	-	7351 - 7399	vulvar carcinoma	Kasher & Roman, 1988
11v	+	6090 - 374	lung carcinoma metastasis	Byrne et al., 1987
16v	-	7598 - 17	cervical carcinoma	Tidy et al., 1989

Figure 5.2

Diagrammatic representation of the sequence alterations located in the URR of several HPV variants, shown in relation to the putative viral enhancer and promoter elements. Deletions and duplications are represented by solid and shaded boxes respectively.



influenced as a result of the deletion and/or duplication of sequences encompassing some of the putative control elements for viral gene expression.

The discovery in genital cancers of HPV 6 and 11 variants with alterations to the URR supports the view that duplication or deletion of URR sequences may be sufficient to confer a higher oncogenic potential on a virus normally associated with low grade lesions (Boshart and zur Hausen, 1986; Rando et al., 1986; Byrne et al., 1987; Kasher and Roman., 1988). Variants of HPV 16 have been found in a variety of lesions, including genital carcinomas. One study, using Southern blotting, found the same HPV 16 URR deletion variant in two independently derived cervical carcinomas inferring that the deleted HPV 16 is a transmissible variant (Tidy et al., 1989) .

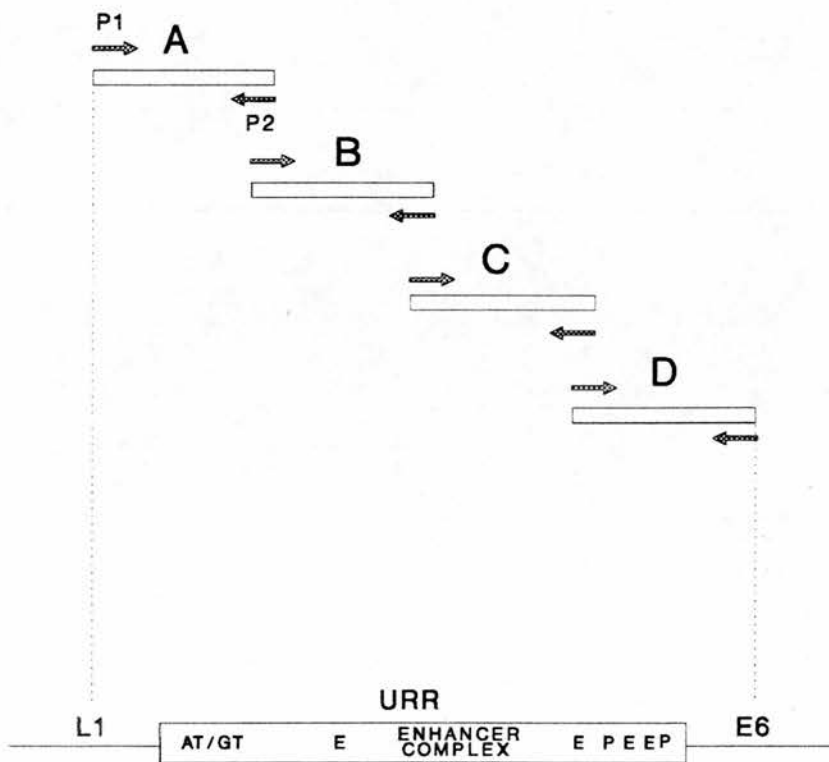
Despite the increasing number of variants being reported and the recent evidence to suggest that they may be transmissible very little is known about their prevalence in cervical lesions with many having come to light as a result of detection assays using PCR or Southern Blotting. In this systematic survey a PCR based assay was specifically designed to detect sequence alterations within the URRs of HPV types 16 and 18 from cervical lesions. The correlation of particular URR sequence alterations with the grade of lesion may offer an explanation as to why some cases of HPV 16 and 18 progress and others persist or regress.

5.2 Assay design

A PCR assay was designed to amplify the entire URR in four contiguous segments (figure 5.3). Small deletions or insertions within the region

Figure 5.3

Arrangement of primer pairs for amplification of segments A-D of HPV 16 and HPV 18. Segments are shown in relation to their location in the HPV genome. Segment A primer 1 sites lie within the L1 gene. Segment D primer 2 sites lie within the E6 gene. All other primer sites lie within the URR. In the URR, E denotes the E2 binding sites and P the promoter elements. The enhancer complex is activated by the binding of cellular factors.



would be seen as a deviation in length from the prototype segment of cloned HPV plasmid DNA when amplified PCR products were electrophoresed on an agarose gel. In order to amplify the URR in four contiguous segments, sequences of the URR and flanking sequences of the L1 and E6 genes of HPV types 16 and 18 were searched for five sites that were approximately evenly spaced and suitable for efficient primers. The number of possible sites was limited owing to the abundance in the URR of unusual sequences - such as those of the AT/GT rich region, likely to have unusual secondary structure - and repeated sequences such as that of the E2 binding palindrome ACCN₆GGT.

Eight oligonucleotide primers were chosen for each HPV type (16 and 18) using UW programs, and were checked for any significant secondary structure, cross hybridisation and 3' complementarity using the approach described in Chapter 3. The %GC content for HPV 16 primers was between 23% and 57%, and for HPV 18 primers between 38% and 65%. Primers chosen were between 20-26 bases in length permitting the use of high PCR annealing temperatures and improving specificity by reducing the incidence of illegitimate binding of primers to near complementary sequences. Primer sequences chosen along with their locations in the HPV genome are detailed in table 5.4.

The PCR assay was optimized for each primer pair using cloned HPV plasmid DNA and an initial primer annealing temperature of 42°C. The optimum annealing temperature was considered to be the highest temperature to allow amplification without reducing the yield of specific amplified product. The optimum temperatures are detailed in table 5.4. Step times for denaturation, primer annealing and extension were varied

Table 5.4

Nucleotide sequence (5' to 3') of the pairs of PCR primers for segments A-D of HPV 16 and HPV 18, their position within the HPV genome according to the EMBL database nomenclature, the lengths of amplified products, optimum annealing temperatures. HPV 16 primer 2(ii) was suitable for use on frozen material, cloned HPV plasmid DNA and DNA extracted from paraffin wax embedded material whereas HPV 16 primer 2 was unsuitable for use on DNA extracted from paraffin wax embedded material.

HPV	SEG	P	SEQUENCE (5' to 3')	POSITION	PRODUCT LENGTH	Ta (oC)
16	A	1	CCT CTA CAA CTG CTA AAC GC	7117-7136	272	50
16	A	2	GTA GCA AAT ATA GTT TAT ATA C	7368-7389		
16	B	1	GTA TAT AAA CTA TAT TTG CTA C	7368-7389	219	46
16	B	2	GAA TGG TTG GCA AGC AGT GCA GG	7564-7586		
16	B	2(ii)	CAA GCA GTG CAG GTC AGG AAA AC	7574-7596	228	46
16	C	1	CCT GCA CTG CTT GCC AAC CAT TC	7564-7586	297	50
16	C	2	CGG TTT GCA CAC ACC CAT GTG CAG	7837-7860		
16	D	1	CTG CAC ATG GGT GTG TGC AAA CCG	7837-7860	213	50
16	D	2	CTG TGG TAA CTT TCT GGG TCG CTC C	121-145		
18	A	1	GCC AAG CGT GTG CGT GTA CG	7104-7123	234	46
18	A	2	CAG TTG TTA CTC ACT AGC CG	7318-7337		
18	B	1	GCC CTA GTG AGT AAC AAC TG	7318-7337	249	46
18	B	2	GTT CAA AAT AGT TAG GAC CAG	7546-7566		
18	C	1	CTG CTC CTA CAT ATT TTG AAC	7546-7566	228	50
18	C	2	GGG ATG ACA GAA TGT TGG AC	7754-7773		
18	D	1	GTC CAA CAT TCT GTC TAC CC	7754-7773	221	50
18	D	2	CAA AGC GCG CCA TAG TAT TG	98-117		

to find the optimum which was 1 min, 2 min, 3 min respectively. Each primer pair generated a fragment of predicted length from cloned HPV DNA (figure 5.5).

In order to achieve optimal separation of amplified products guidelines suggested by Thivierge (1989) were taken into account. Amplified products were electrophoresed on 4% agarose gels (3% Nusieve GTG: 1% Seakem GTG) for segments A-D and on 1% agarose gels (Seakem GTG) for the entire URR, for 8-10h at 100mA along with a DNA size marker (MspI digest of pBR322, Biolabs or 1kb ladder, Gibco-BRL). The length of the electrophoretic gel, the grade of agarose and the DNA size marker were such that sequence alterations (duplications or deletions) in segments A-D of between 10-20 bp in length should have been detectable as indicated by clear separation of 142/154 and 200/220 bp fragments of the 1kb ladder.

The assay was applied to 2 cases of CIN 1, 12 of CIN 2 and 10 cases of CIN 3 (all frozen material which was used directly without DNA extraction), in addition to, 20 cases of squamous cell carcinoma, 11 cases of adenocarcinoma and 3 adenosquamous carcinomas, processed as paraffin blocks and subjected to DNA extraction as described in Chapter 2. All cases used in the assay had been found to contain HPV DNA of types 16 or 18 in the PCR detection assay described in Chapters 3 and 4.

5.3 Results

In a total of 24 cases of CIN and 34 cases of cervical carcinoma no sequence alterations in the HPV URR were found (figures 5.6 and 5.7.1-8).

Figure 5.5

Agarose gel electrophoresis of amplified DNA using cloned HPV plasmid DNA as template and primers pairs for HPV 16 (a) segment A (fragment length 272bp); (b) segment B (228bp); (c) segment C (297bp); (d) segment D (213bp); and HPV 18 (e) segment A (234bp); (f) segment B (249bp); (g) segment C (228bp); (h) segment D (221bp).

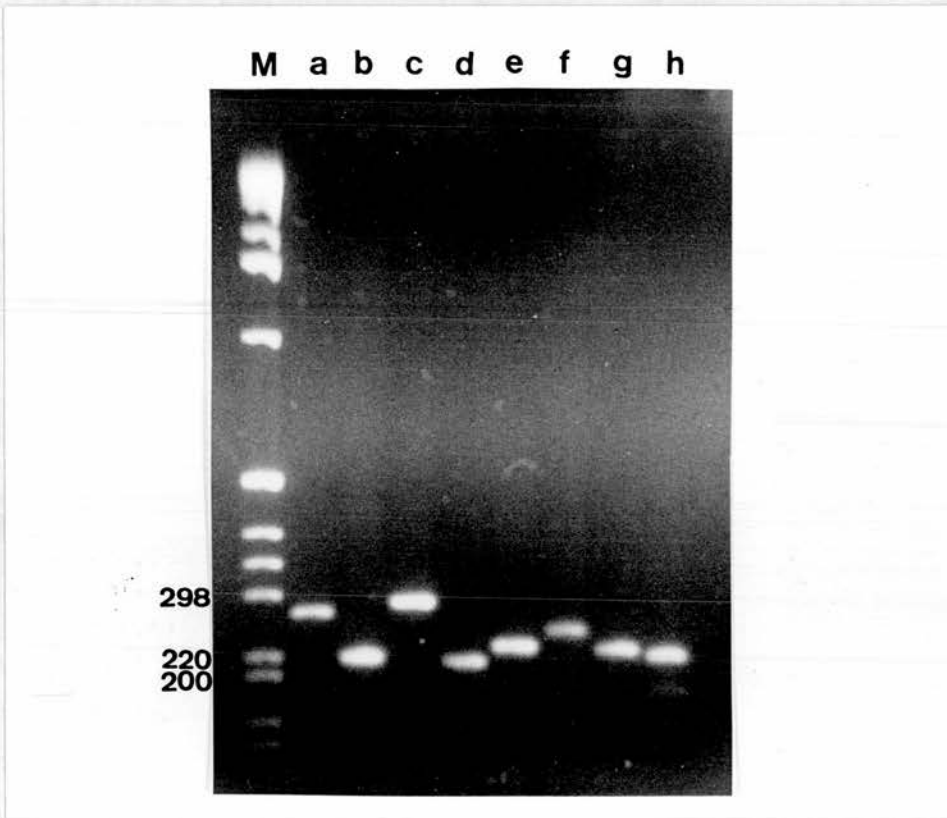


Figure 5.6

Agarose gel electrophoresis of amplified DNA from 7 cases of CIN using frozen sections as a template and PCR primers for HPV 16 segment A (a-g; band B). Included are a cloned HPV plasmid DNA positive control track (P), a template free negative control track (N) and a MspI digest of pBR322 DNA marker track (M). Additional bands visible on the gel (A, C-E) are believed to be "ghost bands" arising from non-specific annealing of primers to near complementary sequences (see text for details).

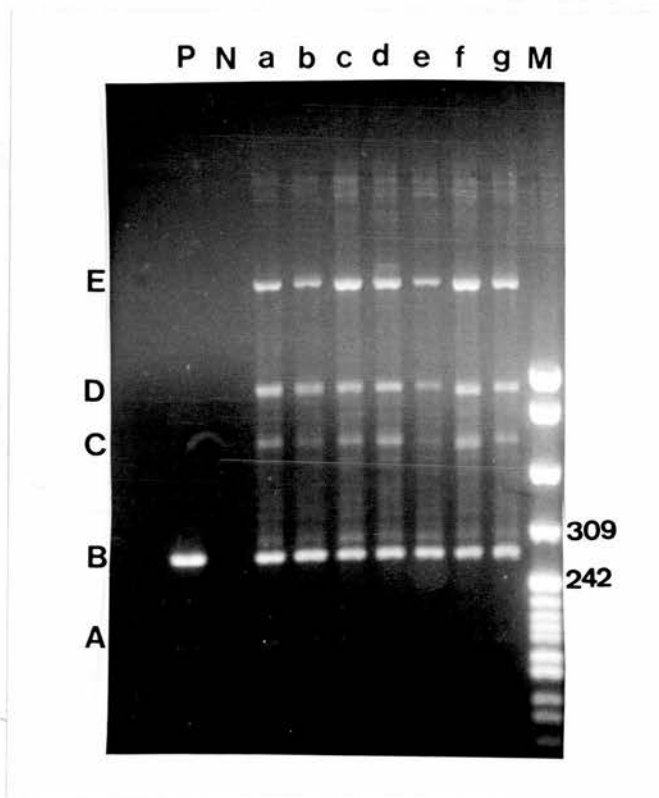
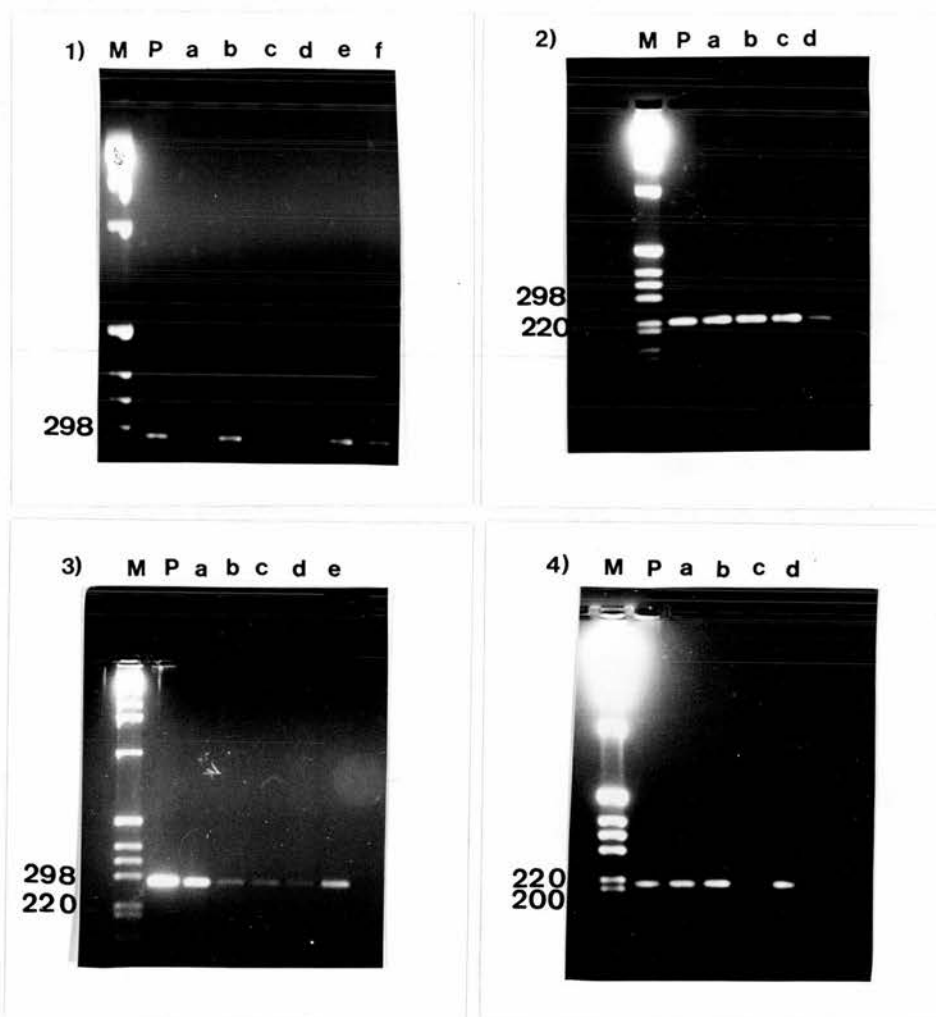
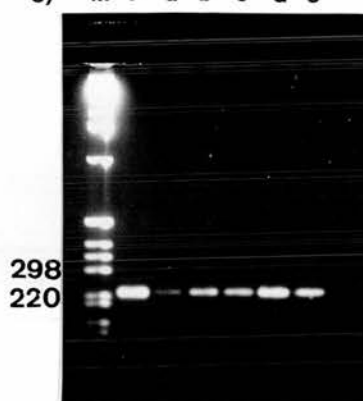


Figure 5.7

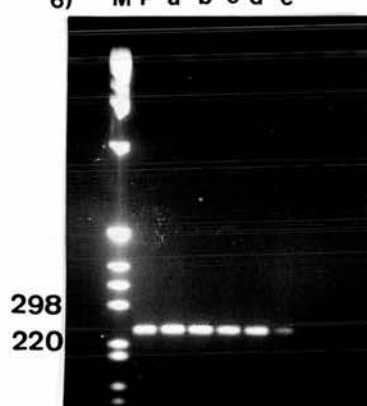
Agarose gel electrophoresis of amplified DNA from cases of squamous carcinoma (a-f) using extracted DNA from paraffin wax embedded tissue as template and primers for HPV 16 segment A (1), B (2), C (3), D (4) and HPV 18 segment A (5), B (6), C (7) and D (8). Included are template free negative control tracks (N), cloned HPV plasmid DNA positive control tracks (P) and 1kb or MspI digest of pBR322 marker tracks (M)



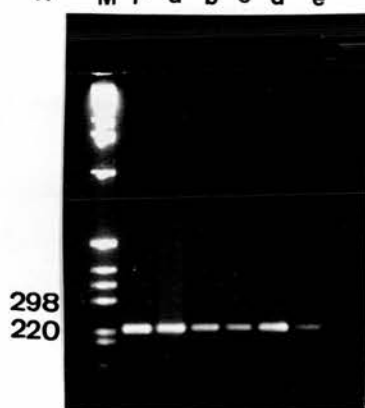
5) M P a b c d e



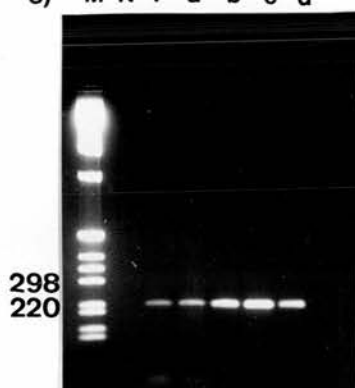
6) M P a b c d e



7) M P a b c d e



8) M N P a b c d

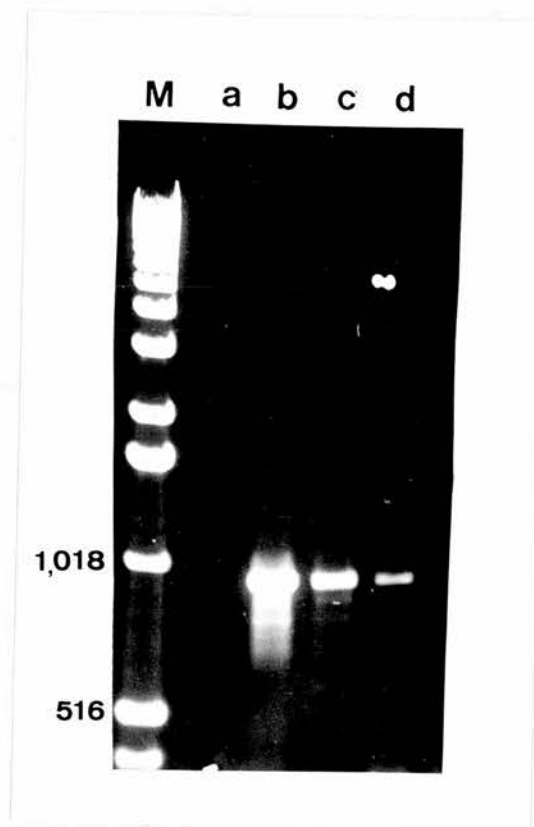


When the assay was applied directly to frozen material, PCR artefacts known as "ghost bands" - which can occur as a result of non specific primer annealing to near complementary sequences - were identified on the agarose gels (figures 5.6). In an attempt to reduce the incidence of these ghost bands; step times for primer annealing and extension were reduced. These measures were unsuccessful. Raising the annealing temperature is often a useful way of improving PCR stringency however the T_a was already as high as primer-target binding stringency would allow. Cervical biopsies were too small to permit the use of DNA extraction procedures. To alleviate this problem, HPV DNA of frozen cases was subjected to additional amplification of the entire URR in one segment (figure 5.8) to look for any large sequence alterations, which could be mistakenly identified as ghost bands, prior to amplification of the URR in four segments.

When the assay was applied to extracted DNA from paraffin wax embedded tissue no ghost bands were generated (figures 5.7.1-8), however the primers for HPV 16 segment B (figure 5.3) produced only "primer dimer" PCR artefacts when reaction products were electrophoresed on an agarose gel. To determine which primer was inefficient two PCR reactions were set up: one using segment A primer 1 with segment B primer 2; another using segment C primer 2 with segment B primer 1. Only the latter combination generated an amplified product of predicted length indicating a problem with segment B primer 2. A reaction set up using segment B primers and a lower annealing temperature - as primer sequences were both short and had a low GC content - failed to generate an amplified product of predicted length. A

Figure 5.8

Agarose gel electrophoresis of amplified DNA from three cases of CIN (b-d) using frozen tissue as template and HPV 16 segment A primer 1 with HPV 16 segment D primer 2 (spanning the entire HPV URR). Included is a template free negative control (a) and a 1kb ladder marker track (M).



new primer 2 for segment B was therefore designed and successfully applied to DNA extracted from paraffin wax embedded tissue (seg B, primer 2;ii, table 5.4).

The HPV type specificity of the assay was apparent when applied to a case of double infection (found to contain both HPV 16 and HPV 18). No additional bands were generated when primers for one HPV type were applied to cases containing both HPV types despite differing product lengths of equivalent segments from each HPV type (table 5.4).

5.4 Discussion

It is possible that the oncogenic potential of individual HPVs could be influenced by sequence alterations within the URR. While there have been several reports of the existence of naturally occurring HPV URR mutants isolated from a range of genital lesions little is known of their overall prevalence in cervical lesions. In this study, a PCR based assay was designed specifically to look for sequence alterations within the HPV URR which might influence oncogenic potential and thus contribute to neoplastic progression.

The assay was applied to material from different sources - both frozen material and DNA extracted from paraffin wax embedded tissue. When applied directly to frozen tissue (24 cases of CIN), gel electrophoresis of amplified products showed not only bands of expected length but often several additional bands (figure 5.6). These were judged to be ghost bands (which arise due to non-specific primer annealing), as opposed to true sequence alterations, on the basis of several observations. (1) The additional bands seen in sample tracks were in some instances seen also

in the plasmid control track and therefore were not considered to be deviations from prototype (figure 5.6 (A)). (2) Identical additional bands were seen in many and often all sample tracks (figure 5.6(C, D, E)). While it is possible that these represented very common deviations previous reports would indicate this to be unlikely. (3) Additional fragments at least 100bp longer than predicted fragment length were not found to be congruent with any sequence alterations found when the entire URR was amplified in one segment (figure 5.6(C, D, E)). (4) In every case the most abundant fragment was that of predicted length (figure 5.6(B)).

The abundance of ghost bands in this assay as compared to the previously described detection assay when applied directly to frozen tissue may be due to the shorter primers used in the URR assay. Shorter primers require lower annealing temperatures and thus the specificity of the assay is reduced. The length of the primers for the URR assay was restricted owing to the abundance of unusual sequences within this region making it difficult to find sequences long enough to minimise ghost band formation.

While it is possible that these additional bands may represent sequence duplications within the URR it was thought to be unlikely. The matter could have clarified by Southern hybridisation of the agarose gels using an HPV type and segment specific oligoprobe probe which would not hybridise to illegitimately amplified genomic sequences.

When the assay was applied to extracted DNA from paraffin wax embedded cervical carcinomas one primer pair failed to generate a band

of expected length in any of the cases examined. One additional fragment approximately the length of the two primers suggestive of a primer dimer PCR artefact was generated. Conditions thought to favour the formation of primer dimers include 3' complementarity between primer pairs, or high primer concentration or low starting template DNA concentration. Had primer or template DNA concentration been the problem a similar result using other primer pairs would be expected and this was not the case. Also there was no obvious complementarity between the 3' ends of primers. The failure of previously effective primer pairs when applied to DNA extracted from paraffin wax embedded tissue has been observed by other investigators within our laboratory.

The design of a PCR based assay to detect large scale sequence alterations allowed a large number of isolates to be screened in a relatively short time whereas, the detection of point mutations would have required the use of more lengthy procedures such as asymptomatic PCR and sequencing. Moreover, while point mutations may affect the oncogenic potential of a virus it is easier to speculate as to the possible effects on pathogenicity conferred by the loss or duplication of putative enhancer and promoter sequences such as those of HPV 16v or HPV 6vc.

There have been, as yet, no reports of HPV 18 viral variants thus it is not surprising that no URR mutants were found in the 16 cases containing HPV 18. The negative results for URR mutants in 43 cases containing HPV 16 compares favourably with the findings of a recent study aimed at looking for sequence alterations in the 3' portion of the HPV 16 URR (Chan et al., 1992). In a total of 118 cases of HPV 16 (90 of which were isolated from cervical lesions and smears) only 38 variants were found.

Most of these variants differed by one or several point mutations. It would appear from these results that large sequence duplications or deletions may not be widespread in cervical lesions, however their existence may offer an explanation as to how low risk or episomal HPVs may contribute to the neoplastic process.

CHAPTER 6

DESIGN OF A PCR BASED ASSAY TO DETERMINE THE PHYSICAL STATE OF HPV DNA IN CERVICAL LESIONS

6.1 Introduction

HPV 16 and 18 have been categorised as high risk HPV types on the basis of their prevalence in high grade CIN and cervical cancers as found by others and in chapter 4 of this study and it has been suggested that the presence of these HPV types in cervical lesions may indicate a greater tendency for progression to invasive carcinoma (Lorincz et al., 1987). However, not all lesions containing HPV of types 16 or 18 develop into invasive cancers and it is thought that additional events are required for neoplastic progression. The results of chapter 5 of this study indicate that URR sequence alterations are rare and are therefore unlikely to be useful indicators of those HPV-containing lesions most likely to progress to invasive carcinoma.

The integration of HPV DNA into the host genome has been proposed as an important event in neoplastic progression. The regular pattern of interruption of viral E2 or E1 genes resulting from the integration event disrupts the negative transcriptional control of E6 and E7 transforming genes by the E2 gene products leading to their continuous or high level expression possibly promoting the development of cancer (Arends et al., 1990).

In support of this, HPV DNA has been found integrated into the host genome in many cervical cancers (Lehn et al., 1985; Durst et al., 1985), in carcinoma-derived cell lines (Yee et al., 1985) and experimentally immortalized human keratinocytes (Barbosa and Schlegel, 1989; Woodworth et al., 1989). In benign productive infections the virus is maintained as an episome. Several studies have shown the presence of integrated HPV DNA in some CIN lesions, although there appears to be

little agreement on the stage in the CIN-cancer sequence at which integration occurs. One study of HPV containing CIN lesions and cervical carcinomas found HPV DNA to be episomal in all CIN 1 lesions and integrated in only 5% of CIN 2 and CIN 3 lesions as compared with 81% of carcinomas (Cullen et al., 1991). A study by Lehn et al (1988) found only episomal HPV DNA in CIN 1 lesions whereas 91% of the CIN 2 and CIN 3 lesions contained integrated HPV DNA. Further investigation of the physical state of HPV DNA in CIN and cervical carcinomas is required to determine when integration occurs and whether it indicates a greater likelihood for progression to invasive carcinoma establishing its role as a useful prognostic indicator.

Non-isotopic *in situ* hybridisation has been used by some investigators to distinguish between episomal and integrated HPV DNA (Cooper et al., 1991). However, this method relies on subjective interpretation of different staining signal types. The experimental approach employed by most investigators to determine the physical state of HPV DNA in clinical samples is that of restriction enzyme mapping with subsequent Southern blot hybridisation. In cases where the results from these techniques are difficult to interpret two-dimensional gel electrophoresis is often used as a confirmatory measure. One drawback of these techniques is that they require large amounts fresh tissue from which to extract DNA. Many CIN 2 and CIN 3 lesions affect only small regions of cervical epithelium. PCR detection of HPV DNA can be applied to small clinical samples but presently used assays cannot differentiate between integrated and episomal HPV DNA.

In this study, an anchor PCR (A-PCR) based assay was designed and

developed for use in determining the physical state of HPV 16 DNA in small samples of cervical lesions. The assay was aimed at distinguishing the integrated form of HPV DNA from the episomal monomeric and multimeric forms. Episomal multimers can occur as head to tail repeats which may be intact or show deletions and duplications (Choo et al., 1989; di Luca et al., 1989). Although relatively uncommon, episomal multimers can complicate the interpretation of restriction enzyme patterns from integration assays based on Southern blotting (Cullen et al., 1991). Some lesions may have combinations of integrated and episomal forms.

6.2 Assay Design

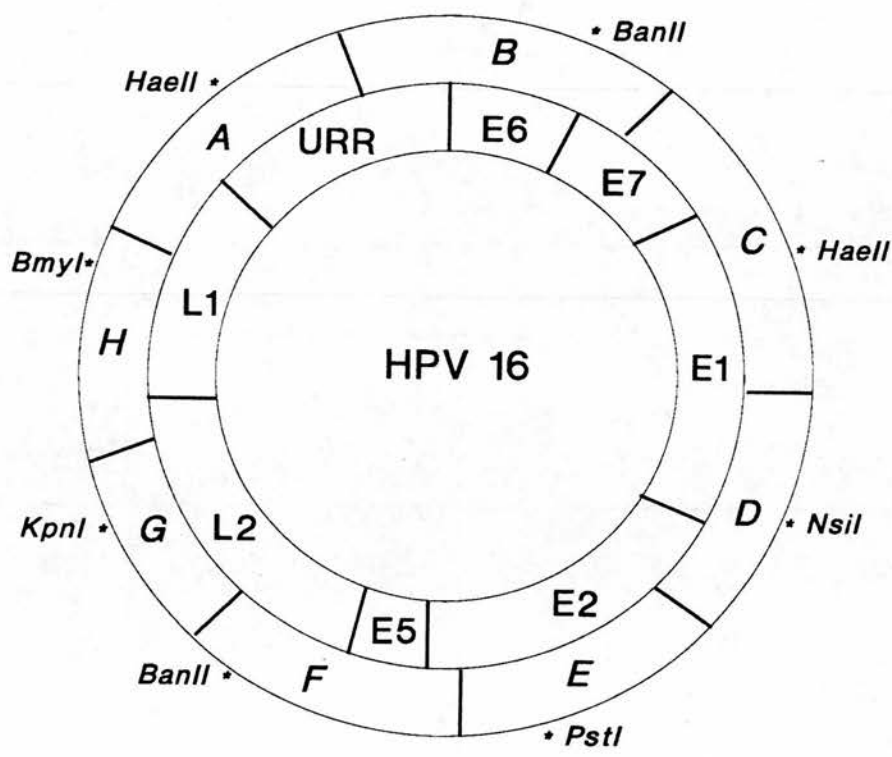
The assay can be divided into two separate stages: initial PCR analysis to detect the presence of the entire viral genome using standard procedures, followed by an A-PCR based analysis developed specifically for discriminating between integrated and episomal HPV DNA.

6.2.1 PCR Analysis

For the primary analysis, PCR is used to amplify the entire HPV genome in eight contiguous segments (A-H, fig 6.1), each of approximately 1kb in length. Amplified products are electrophoresed on 1% agarose gels and the results interpreted as follows. If only integrated HPV DNA is present, at most seven of the eight reactions (and possibly fewer) will result in amplified products of expected length. This being the case the site of integration in the non-amplifiable segment could be confirmed by subsequent A-PCR analysis applied to that segment (see below). If an irregular multimer (with deletions or duplications) was present, PCR amplification would be likely to generate some additional fragments of unexpected length. This could be confirmed by Southern blotting using

Figure 6.1

Segments A-H of HPV 16 shown in relation to the viral ORFs. A suitable A-PCR restriction site is shown for each segment.



specific oligoprobes from each of the segments of the HPV genome, or by cloning and sequencing amplified fragments. If episomal (monomer or intact multimer) HPV DNA is present a fragment of predicted length for each segment A-H will be generated. This may mask any integrated HPV DNA which might be present within the same clinical sample. Thus, any such case found to contain episomal HPV DNA would be subjected to A-PCR for each segment A-H (see below).

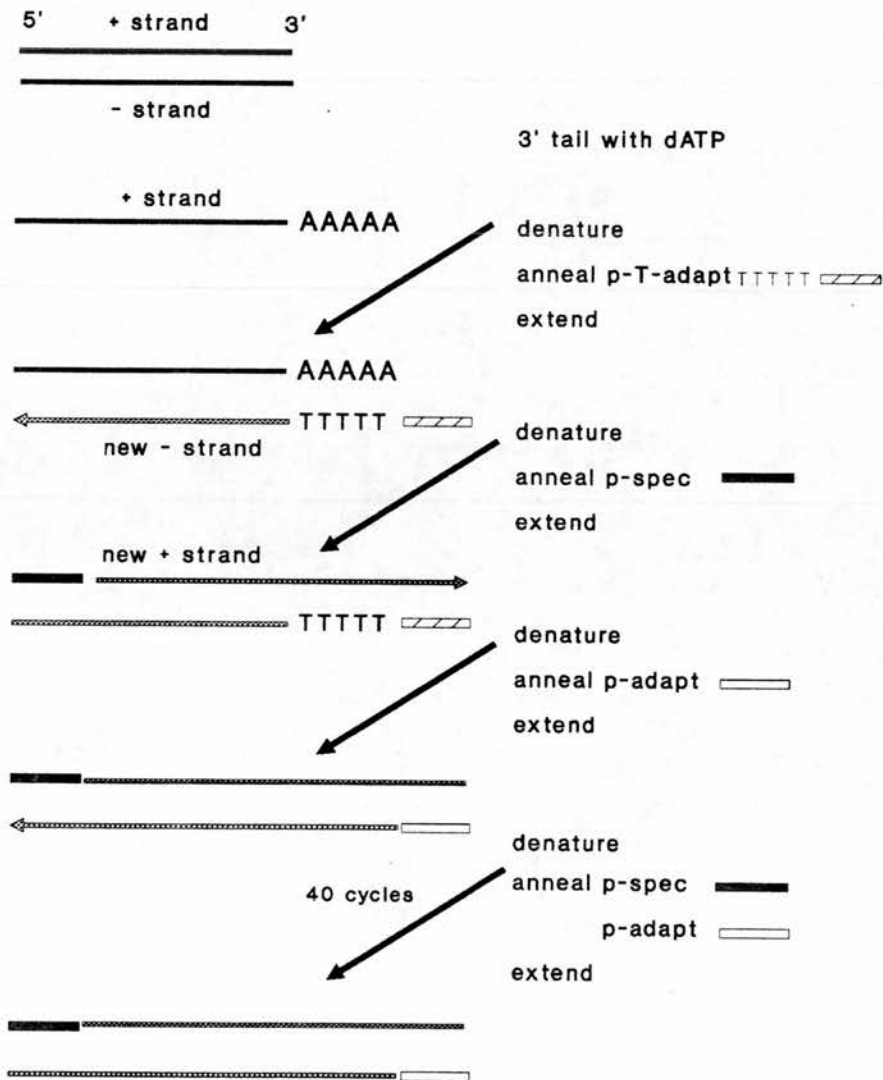
6.2.2 Anchor-PCR Analysis

A modified Anchor-PCR method can be used to engineer a primer site (**p-adapt**) at a known restriction enzyme cutting site and allow amplification between the restriction site and a known specific primer site (**p-spec**). This allows amplification of a viral-host DNA junction when a virus integrates randomly within the host genome because a p-spec site is always available within the integrated viral genome.

Template DNA is digested with an appropriate restriction enzyme (see below) and 3' ends are tailed with dATP (figure 6.2). PCR amplification is subsequently achieved by virtue of three primers: a viral sequence specific primer (**p-spec**), a unique sequence 22 base primer (**p-adapt**) and a hybrid primer consisting of oligo-dT (17 T nucleotides) linked to the adapter primer (**p-T-adapt**). The hybrid primer (**p-T-adapt**) anneals to the 3' poly A tail. This is required for the initial cycles of amplification. Subsequent cycles of amplification can be achieved using the adapter primer (**p-adapt**) and gene specific primer (**p-spec**). As the hybrid primer (**p-T-adapt**) is only needed for the start of the reaction, it is used at a lower concentration than the other two primers.

Figure 6.2

Schematic representation of the A-PCR protocol. PCR amplification is achieved through use of three primers p-spec, p-T-adapt and p-adapt. Only one of the original DNA strands (the positive strand in this example) is amplified.



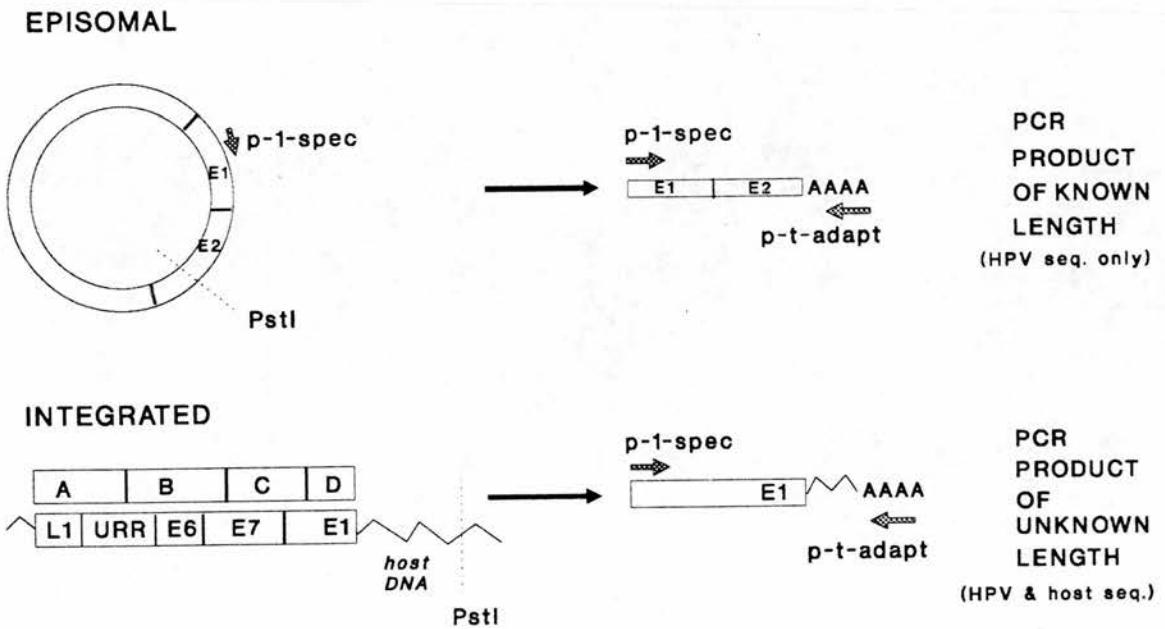
This anchor-PCR analysis is used to investigate which particular segments of the HPV genome (A- H) are disrupted by integration. Initial PCR analysis will give an indication as to the segment in which integration is most likely to have occurred, provided only integrated HPV is present in the sample and anchor-PCR analysis would be carried out on these segments only. However, if episomal DNA is present in the sample, anchor-PCR would have to be carried out for each segment (A-H) starting with the most likely sites for integration that is E2, E1 and L2.

Template DNA would be digested with an enzyme which cuts within the segment adjacent to the segment of interest. For example if investigating the possibility of integration within segment D, (which covers E1 and E2 ORFs) template DNA would be cut within segments E or C (using PstI or HaeII respectively). The 3' ends of the DNA would then be tailed with dATP. Segment D primer 1 (p-1-spec) would be used if cutting in E (with PstI). Alternatively, segment D primer 2 (p-2-spec) would be used if cutting in C (with HaeII)(figure 6.1). As restriction enzymes will cut at known sites within episomal DNA, if only episomes are present, gel electrophoresis of amplified products will show fragments of known predicted lengths. In contrast, if integrated DNA is present then amplified fragments will be of different unknown lengths depending on the cutting site in adjacent host DNA (figure 6.3). Southern blot analysis of amplified products using HPV type specific probes could then be used to confirm which of the fragments contain HPV DNA.

The use of the A-PCR based assay offers the opportunity to gather direct evidence of viral integration via amplification of viral-host DNA junctions which could be subsequently cloned and sequenced if desired,

Figure 6.3

Schematic representation of A-PCR assay for integration within the HPV 16 segment D (E1, E2 genes). PstI will cut episomal HPV DNA at a known site and genomic DNA adjacent to the integrated HPV genome at an unknown site. A-PCR with p-1-spec and p-T-adapt will generate a product of known length with episomal DNA and a product of unknown length with integrated HPV DNA. That this product contains HPV sequences can be confirmed by Southern blotting.



for example, for investigation of host integration sites.

6.2.3 Primer Design

The entire HPV 16 genome was searched for eight suitable evenly spaced sites with sixteen PCR primer sequences to be used both in the initial PCR analyses and also with adapter and hybrid primers in the subsequent anchor-PCR analyses. Non-specific amplification can be minimised if all the primers in a particular reaction have similar melting temperatures. Thus HPV specific primers were chosen which had a similar length and GC content to the adapter primer. HPV specific primers were between 21 and 27 bases in length and had between 33% and 56% GC content, whereas the adapter primer was 22 bases long and had a GC content of 54%. All primers were checked for any significant secondary structure as predicted by the UW programs FOLD and SQUIGGLES and the program FIND was used to exclude the possibility of exact or related (with up to 5 mismatches) primer sequences occurring elsewhere within the same or other HPV types. The "unique" sequence chosen for the adapter primer was an extended version of an adapter sequence described by Frohman (1990) for use in a PCR protocol for rapid amplification of cDNA ends (RACE) on which this A-PCR method is based. Sequences chosen for HPV type specific primers and adapter primer are shown in table 6.4.

6.2.4 Optimization of the conditions for Initial PCR analysis

The standard PCR assay was optimized for each of the HPV type specific primer pairs using the cloned HPV16d plasmid DNA (figure 6.5). This plasmid contains a head to tail tandem duplication of prototype HPV 16 cloned into pBR322 at the BamHI site. The use of this plasmid allowed the amplification of all segments (A-H) of the HPV 16 genome including

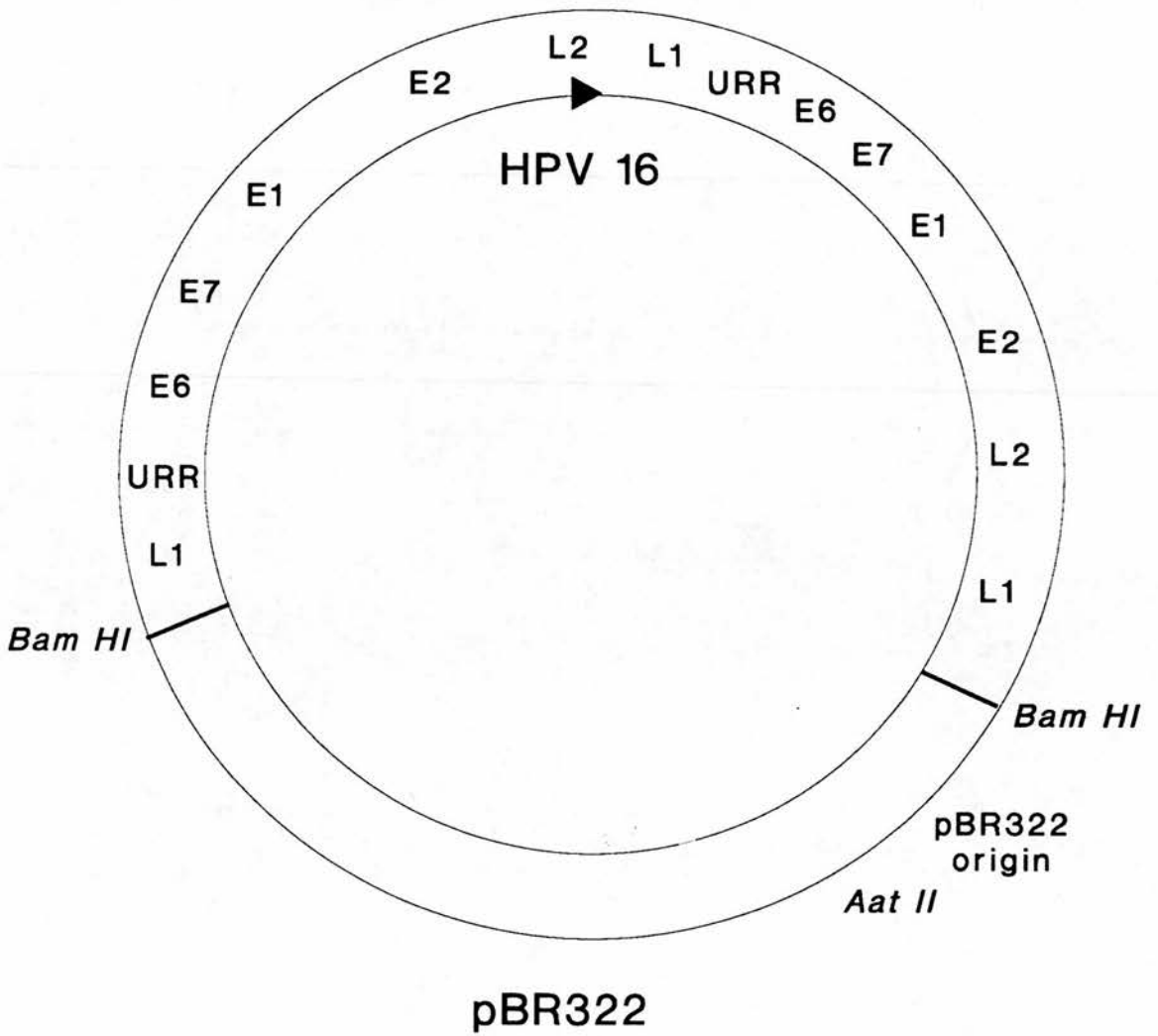
Table 6.4

Nucleotide sequence (5' to 3') of the pairs of primers for segments A-H of HPV 16, their position within the viral genome according to the EMBL database nomenclature, length of amplified product and the open reading frames covered by each segment.

SEG	PRIMER	SEQUENCE (5' to 3')	POSITION	PRODUCT LENGTH	ORFs COVERED
A	1	CAT TAT GTG CTG CCA TAT CTA CTT GAG	6664-6690	933	L1, URR
A	2	GAA TGG TTG GCA AGC AGT GCA GG	7574-7596		
B	1	CCT GCA CTG CTT GCC AAC CAT TC	7574-7596	1133	URR, E6, E7
B	2	CTT CCA AAG TAC GAA TGT CTA CGT G	778-802		
C	1	CAC GTA GAC ATT CGT ACT TTG GAA G	778-802	1127	E7, E1
C	2	GTT GTA ATA CTG TTT GTC TTT GTA TCC	1879-1905		
D	1	GGA TAC AAA GAC AAA CAG TAT TAC AAC	1879-1905	1013	E1, E2
D	2	GCC TTG TAA TAA ATA GCA CAT TCT AG	2866-2891		
E	1	CTA GAA TGT GCT ATT TAT TAC AAG GC	2866-2891	978	E2
E	2	CAT AAA TCC AGT AGA CAC TGT	3823-3843		
F	1	ACA GTG TCT ACT GGA TTT ATG	3823-3843	819	E2, E5, L2
F	2	GTA ATA CTA AAT CCT GAT ACA TCT G	4617-4641		
G	1	GAT ACT GGG ACA GGA GGC AAG TAG AC	4617-4641	1084	L2
G	2	CAA CCT TAG ATA CTG GGA CAG GAG GCA	5674-5700		
H	1	GTC TAC TTG CCT CCT GTC CCA GTA TC	5667-5692	1024	L2, L1
H	2	CTG AAG TAG ATA TGG CAG CAC ATA ATG	5667-5692		
ADAPTER		GAC TCG AGT CGA CAT CGT ATG C			

Figure 6.5

Diagram of the HPV 16d plasmid which contains two tandem copies of HPV 16 cloned into pBR322 at the BamHI site.



segment H which cover the BamHI site in the L1 ORF used to clone a single copy of the HPV 16 genome. The annealing temperatures were optimized by setting up separate PCR reactions and increasing Ta by increments of 2°C starting at 50°C until yield of desired amplified product was optimised. A Ta of 56°C could be used successfully with all primer pairs (figure 6.6A).

The primary standard PCR assay was applied successfully to extracted DNA from two cases of cervical carcinoma processed in paraffin wax. For both cases all eight reactions generated fragments of predicted length indicating the presence of episomal HPV DNA (figure 6.6B).

6.2.5 Selection of Restriction Enzyme Sites

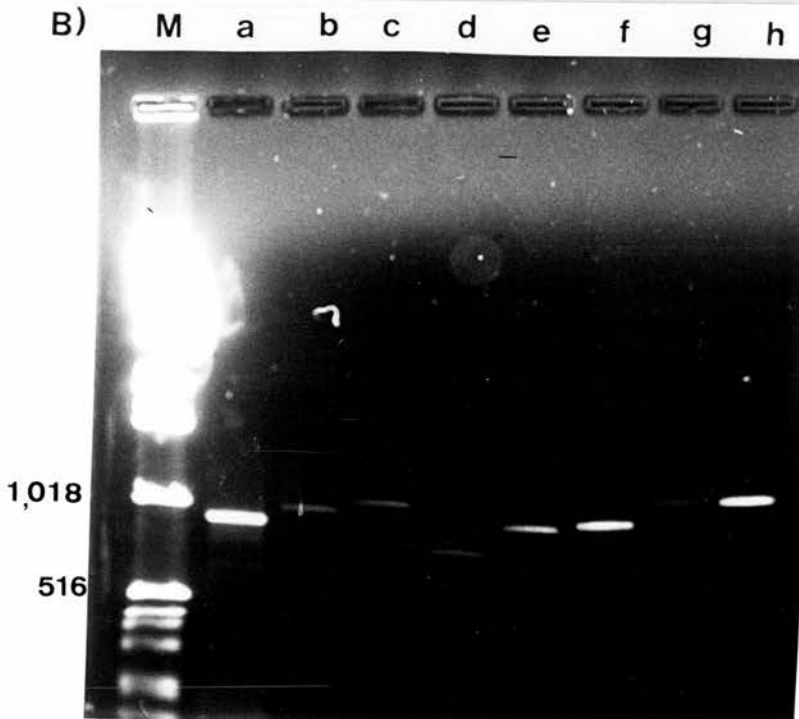
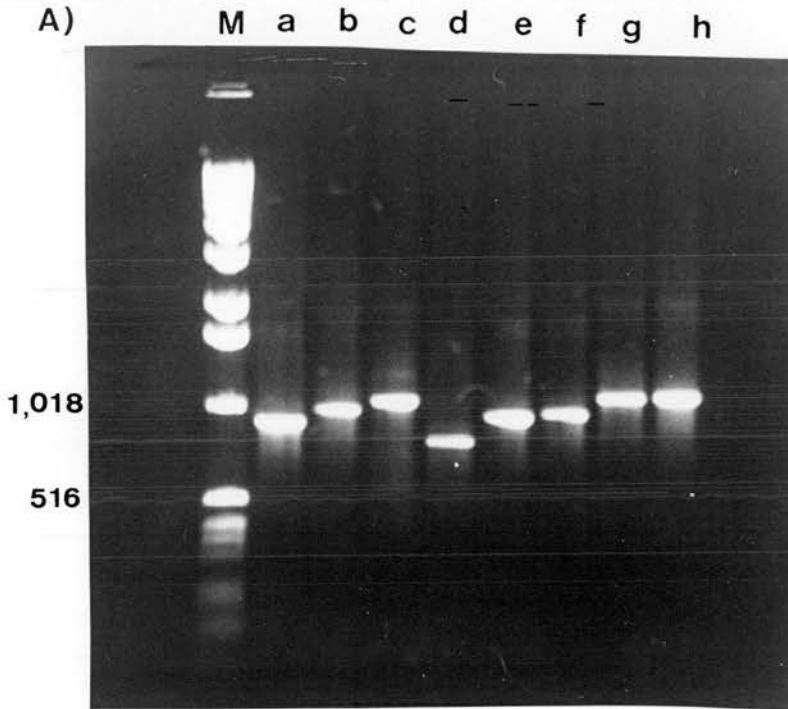
The UW program MAPLOT was used to search the entire HPV genome for suitable restriction sites for use in the anchor-PCR analysis. To be of use restriction enzymes had to fit several criteria: to create a 3' overhang to maximise the efficiency of subsequent TdT tailing; to have at most a six base, but preferably a four base, recognition sequence in order that host DNA is likely to be cut at frequent enough intervals to allow PCR amplification of products less than 2.5kb - the empirical maximum for efficient PCR (Saiki, 1989); to cut within one segment (A-H), but neither of the two adjacent segments. Several possible sites were found for each segment the best of which are shown in figure 6.1.

6.3 Results

The anchor PCR method including restriction enzyme digestion and TdT tailing was validated using HPV 16 DNA cloned into pBR322 at the BamHI site (pHPV16) to mimic integration within the viral L1 gene

Figure 6.6

Agarose gel electrophoresis of amplified DNA from (A) cloned HPV 16 plasmid DNA and (B) a case of adenocarcinoma using primer pairs for HPV 16 segments A (a;length 933bp), H (b;1024bp), G (c;1084), F (d;819bp), E (e;978bp), D (f;1013bp), C (g;1127bp) and B (h;1133bp). Both (A) and (B) include a 1kb ladder marker track (M).



(segment H).

Firstly, plasmid DNA was linearised by digestion with restriction enzyme AatII which cuts pBR322 once at position 4290 but will not cut HPV DNA, generating one fragment approximately 12kb in length upon agarose gel electrophoresis (figure 6.7). The 3'end labelling with TdT was then validated using AatII restricted pHPV16 DNA and P³²-labelled dATP. Tailed fragments were electrophoresed on agarose gels and successful 3' poly A tailing confirmed by autoradiography (figure 6.8)

Tailed AatII digested pHPV16 DNA and HPV specific primer (p-1-spec) for segment H were used to develop the anchor-PCR protocol. The RACE protocol (Frohman, 1990) which is very similar in principle to the anchor-PCR method was used as a starting point. However, the use of this method did not result in the amplification of a specified fragment of predicted length. Multiple modifications were made to the method. These involved increasing denaturation, primer annealing and extension temperatures, reducing dNTP concentration, omitting DMSO, decreasing hybrid primer (p-T-adapt) concentration with respect to adapter (p-adapt) and specific (p-spec) primers. The protocol as described in Chapter 2 resulted in successful amplification of the specified fragment (consistent with the predicted length of 1028bp) detected by agarose gel electrophoresis (figure 6.9).

6.4 Discussion

A PCR based assay was designed for the determination of the physical state of HPV DNA in small clinical samples such as those from punch biopsies of CIN. The first stage of the assay was designed to detect the

Figure 6.7

Agarose gel electrophoresis of the restriction enzyme digest of cloned HPV 16 plasmid DNA using AatII. A single fragment, 12kb in length is generated (b, c; in duplicate). Included are an uncut HPV 16 plasmid DNA track (a) and a 1kb ladder marker track (M).

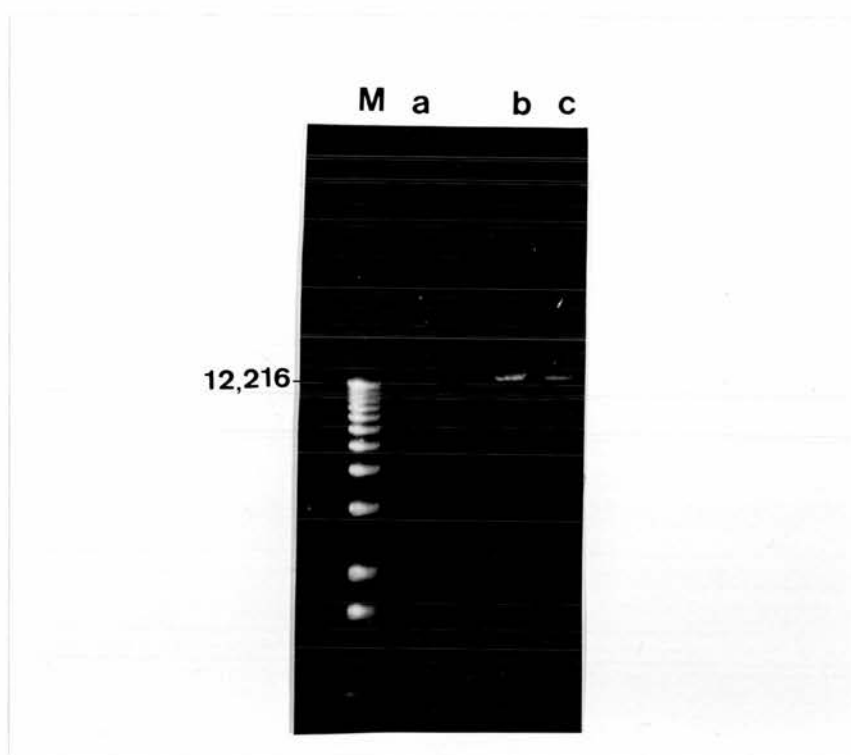


Figure 6.8

Autoradiograph of AatII restricted HPV 16 plasmid DNA, 3' poly A tailed with [P^{32}] labelled dATP (in duplicate; a, b).

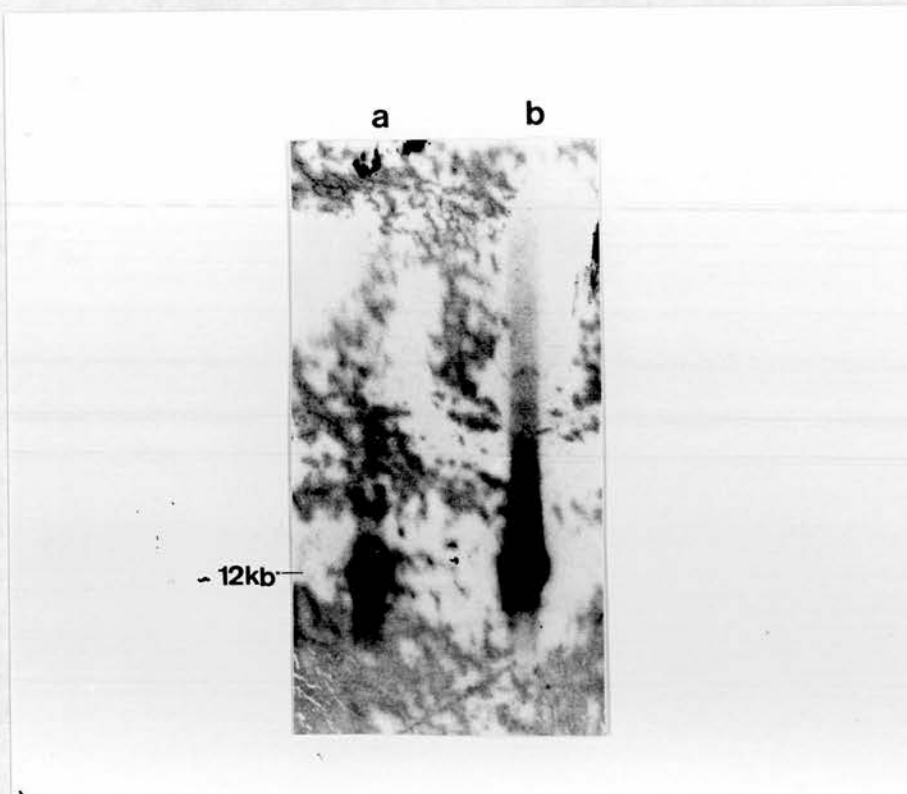
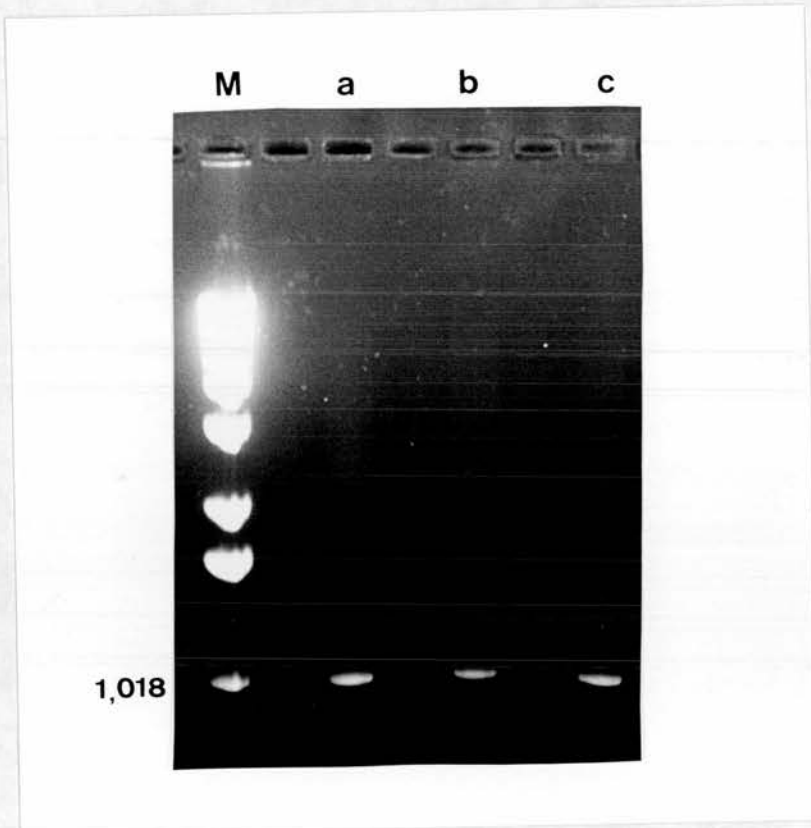


Figure 6.9

Agarose gel electrophoresis of cloned HPV 16 plasmid DNA restricted with AatII, 3' poly A tailed and amplified by A-PCR using p-spec-1 for segment H (in triplicate; a, b, c). The size of the fragment generated is consistent with the predicted length of 1028bp (from p-spec-1 site for segment H in HPV 16 to the AatII site in pBR322. Included is a 1kb ladder marker track (M).



presence of monomeric and irregular multimeric episomes and also to indicate possible presence of integrated HPV DNA if no episomes were present. The subsequent A-PCR stage of the assay was designed to detect the presence of integrated HPV DNA.

The first stage of the assay has been successfully applied to cloned HPV plasmid DNA and to clinical samples in the form of DNA extracted from paraffin blocks of two cervical carcinomas. The absence of ghost bands generated in this assay when applied to clinical samples is indicative of its high specificity which could be attributed to the primer design (primers were of at least 21 bases in length and had a %GC content of 33%-56%) which allowed the use of high annealing temperatures .

The high specificity of the HPV primers was particularly important to the A-PCR analyses where the second primer is always the non-specific oligo T-adapter hybrid (p-T-adapt). In order to maximise further the specificity of the A-PCR assay an adapter primer was designed which was similar to that described by Frohman for the RACE protocol with the addition of 6 bases to the 3' end which allowed the use of higher annealing temperatures. Also taken into account was the nucleotide used in the tailing reaction; dC and dG were avoided as homopolymers of these nucleotides bind with a much higher affinity than those of dA or dT and therefore much shorter stretches of dCs or dGs than dAs or dTs are required in the template DNA sequence for non-specific binding of the complementary homopolymer-adapter hybrid primer. In order to improve the efficiency of the tailing reaction from 20% to up to 90%, enzymes were used which cut to give a 3' overhang, as opposed to a 5' overhang or blunt ends.

The A-PCR secondary step of the assay was successfully applied to cloned HPV 16 plasmid DNA. However, it is likely that the method described in Chapter 3 would have to be optimized and refined for use on clinical samples. Frozen sections would be preferable to paraffin wax processed tissue as a template source. It is possible that the application of the A-PCR method to DNA extracted from paraffin wax embedded tissue may prove problematic. Several investigators have reported problems with certain restriction enzymes inefficiently digesting DNA from tissue processed in this way. Such DNA preparations may not be in a condition to allow PCR amplification over a distance greater than 1kb due to fixation and extraction procedures (Wright and Manos, 1990).

Once optimized for use on frozen sections of punch biopsies this assay could offer several advantages over other approaches. The use of a PCR based assay means that only very small amounts of DNA are required and thus the method could be applied to small clinical samples. The approach described here should be less time consuming than those requiring Southern blotting and two-dimensional gel electrophoresis. The A-PCR amplification of integrated HPV DNA would allow the further analysis of viral-host DNA junctions through cloning and sequencing providing direct evidence for integration of HPV into the host genome and permitting investigation of possible preferred host sequences for viral integration. Finally, this assay could offer information about the region of the HPV genome disrupted during integration. Such information could be valuable in the study of possible effects of integration on viral gene expression. Finally, this method could be used to survey large numbers of cases of CIN 2, CIN 3, localised regions of microinvasive carcinoma

and established invasive carcinoma, to generate accurate data on the frequency of HPV integration in these lesions which could be used to establish its key stage of action in the CIN-cancer sequence.

CHAPTER 7

DISCUSSION

The prevalence of HPV DNA in normal control samples has been a subject of debate for some years. Widely differing prevalences have been reported and both the source of controls and the detection methods used have been suggested as possible reasons for the discrepancies (Munoz et al., 1988). Another additional explanation for the variation in HPV prevalence is highlighted in a study by Schneider et al (1992) where the repeated evaluation of HPV 16 status in cervical swabs from young women with a history of normal Papanicolaou smears revealed temporal fluctuation in individual HPV positivity. Smears from 21 women were analyzed for HPV DNA at intervals of five weeks for one year using PCR. Prevalence estimates per visit varied between 14.3% and 33.3%. The cumulative prevalence was 66.7% as fourteen women were positive at least once. It should also be borne in mind that negative control samples are usually taken from one region of the transformation zone and it is possible that repeated sampling of different regions around the transformation zone may influence HPV prevalence rates.

In this study, no HPV DNA was found in any of the normal control samples. This result should be interpreted with caution. It does not suggest that there are no cases of HPV infection in the normal population only that the prevalence of HPV infection in the normal population is likely to be lower than recent studies have indicated (Young et al., 1989). It is expected that the application of this detection method to further control samples obtained at forensic autopsy would produce HPV prevalence values similar to those of a recent study (3.5%) where controls were selected on a random basis (Van de Brule et al., 1991).

The majority of studies investigating HPV prevalence have chosen

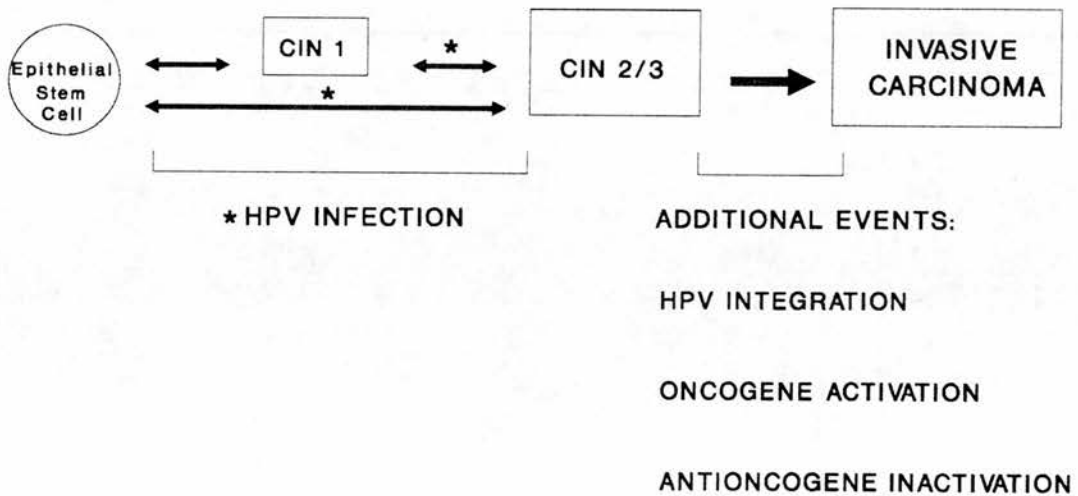
controls designed to be representative of the general population. The question of whether controls should instead reflect a population exposed to the same risk factors as the cases with abnormal cytology has seldom been addressed. Such a study would require a control group matched to the test group (those with CIN and cervical carcinoma) for factors such as age, smoking and sexual history, including age at first intercourse and number of sexual partners.

This study was designed to provide accurate data on HPV prevalence for each stage of the CIN-cancer sequence so as to determine the stages in the sequence at which HPV may make important contributions. The results demonstrate a prevalence jump of 25% to 60% from CIN 1 to CIN 2/3 that can be interpreted in several ways. It is possible that CIN 1 may be induced by factors other than HPV infection, and thus may not always represent a precursor stage to invasive cervical cancer. Furthermore, in the CIN-cancer sequence HPV 16/18 infection may be responsible for progression from CIN 1 to CIN 2/3 or possibly from normal tissue directly to CIN 2/3 (figure 7.1).

Anderson et al (1991) have assessed the current system of nomenclature for cervical epithelial abnormalities. Under the present system, diagnostic consistency at the less severe end of the spectrum is appreciably worse than at the severe end (Ismail et al., 1989). Epithelial changes which do not quite amount to CIN 1 but are not normal may be currently categorised as CIN 1. Anderson et al (1991) recommended the introduction of a new category of epithelial abnormality to encompass those lesions in which a diagnosis of CIN cannot be made with certainty. It is possible that some of the CIN 1 lesions used in this study may fall

Figure 7.1

HPV infection may, in the majority of cases (*), provoke conversion to CIN 2/3 directly from normal tissue by-passing a stable CIN 1 stage, or conversion from CIN 1 to CIN 2/3. Progression to invasive carcinoma may involve integration of HPV DNA into the host genome, oncogene activation and antioncogene inactivation.



into the proposed new category but these are likely to represent only a small proportion of the cases.

It is possible that HPV types other than those investigated in this assay may be responsible for inducing some CIN lesions. Lorincz et al (1987) investigated HPV prevalence in CIN and cervical carcinomas using Southern blotting. Although this method could detect a wide range of HPV types, only HPV 6/11, 16, 18 and 31 were characterized. In CIN 1 lesions they found a 29% prevalence rate for HPV types 6, 11, 16 and 18 as compared with an overall HPV prevalence of 44%. HPV types of low oncogenic potential (analogous with HPV 6 and 11) may be present with increasing frequency toward the less severe end of the CIN continuum.

The results of this study support a role for HPV 16 and HPV 18 in neoplastic progression. These two types were found in 80% of all carcinomas. The remaining 20% may contain HPV types other than those investigated in this study or be genuinely unrelated to HPV infection. Clonal p53 mutations have been found in some HPV- negative cervical carcinomas (Crook et al., 1992). It was suggested that somatic mutation of the p53 gene mediates loss of p53 function in the absence HPV E6. This may explain the apparently worse prognosis of HPV-negative cervical cancers. However, the correlation is not absolute inasmuch as there are HPV-positive cervical carcinomas which have mutated p53 and HPV- negative cervical carcinomas which do not (Fujita et al., personal communication).

There appears to be a relative tropism of HPV 18 for cervical adenocarcinomas which have a poorer prognosis than squamous

carcinomas. This is analogous to the situation described by Stoler et al (1991) for HPV tropism in the clinically aggressive small- cell neuroendocrine carcinomas of the cervix: HPV 18 was found in 78% of 18 of these carcinomas whereas HPV 16 was present in 2 (11%) and 2 cases contained neither type.

In this study, HPV 18 was detected in a much lower percentage of CIN (8%) than squamous carcinoma (23%). This pattern was different from that of HPV 16 which was found in in 38% of CIN lesions and 58% of squamous carcinomas. These prevalence data for precursor lesions and cancers may be expressed as a single index, for purposes of comparison of viral types, by the calculation of Cancer/CIN ratios. HPV 16 had a cancer/CIN ratio of 1.5 and HPV 18 ratio of 2.9, suggesting that HPV 18 is associated with a higher rate or risk of transition to cancer. Such ratios are influenced by variations in both numerator and denominator, and small changes in either may result in larger changes in the overall ratio, particularly if the data set is small. Due to the limited number of CIN lesions and carcinomas used in this study, the Cancer/CIN ratios must be interpreted with caution. Ideally, calculation of such ratios would be used to analyze results from a much larger data set. Such a data set is unlikely to be available to many researchers and the differences in individual detection assays makes the concept of a cumulative data set impractical. Taking these qualifications into account, it appears that HPV 18 was found to associate with a more advanced cervical neoplasia than HPV 16.

These results are strengthened by several reports of an association of HPV 18 with a more aggressive or rapidly progressive type of carcinoma. Walker et al (1989) reported a significantly higher recurrence rate

associated with HPV 18- containing carcinomas as compared to those with HPV 16, irrespective of tumour grade or histological type. Furthermore, patients with HPV 18 containing tumours were more likely to give a history of recent normal Papanicolaou smears than those whose tumours contained HPV 16. Kurman et al (1988) found HPV 18 in a much higher percentage of cervical carcinomas (22%) than in CIN lesions (3%) compared to the prevalence of HPV 16 in 41% of cancers and 37% of CIN lesions. Similarly, a recent study by Lorincz et al (1992) found HPV 18, 45 and 56 in 27% of 153 cancers but only 6.5% of 261 high grade CIN lesions. HPV 16 was found in 47% of both high grade CINs and cancers. Although the possibility exists that HPV 18 associated cancers may evolve by a different pathway than via CIN, there are important clinical implications of a possible subset of lesions with a capacity for rapid progression.

The assay used in this study was designed specifically to look for HPV types of known oncogenic potential in cervical lesions and carcinomas. Until recently there was little evidence about the oncogenic potential of HPV types other than HPVs 6, 11, 16 and 18 and to a lesser degree types 31, 33 and 35. Prospective studies have now been conducted in order to establish the relationship between particular HPV types and cervical neoplasia (Lorincz et al., 1992). Graphic analysis of HPV prevalence ratios at specific points in the diagnostic spectrum defined four risk categories: (1) low risk (HPVs 6/11, 42, 43 and 44); (2) intermediate risk (HPVs 31, 33, 35, 51, 52 and 58); (3) high risk/HPV 16; and (4) high risk/HPV 18 (HPVs 18, 45, and 56). Particularly concerning, in view of the proposed propensity of HPV 18 lesions for rapid progression, are the high risk/HPV 18- related types (HPVs 45 and 56). With this in mind, a

useful continuation of the present study would be type-specific PCR detection of some of the less common genital HPV types (in particular high risk HPV 18-related types 45 and 56 and intermediate risk HPV types 31, 33, 51, 52 and 58) in different stages of cervical neoplasia. As in the present study, type specific primers would be preferable to consensus primers as the latter may fail to detect all HPV types within a lesion as a result of preferential amplification of the type with a higher concentration of viral DNA.

While HPV types 16 or 18 may be involved in progression to high grade CIN, there are cases which regress and some which persist. Therefore, this thesis looked at two further events which may contribute to the development of invasive carcinoma - URR alterations and viral integration.

PCR assays were designed to look specifically for small sequence alterations in the URRs of HPV 16 and 18 found in cervical lesions. The results of this study suggest that viral variants, such as those previously described, are likely to be rare, although it is noted that regional variations may exist. It is unlikely therefore that viral variants with large scale URR alterations contribute to a large proportion of cervical cancers. A study by Chan et al (1992) has shown that by far the most common sequence alteration in the URR is the point mutation. Such small changes in non-conserved and non-protein-coding sequences are unlikely to be of great functional significance. Detection of point mutations could be achieved by sequencing, although this would be both laborious and time consuming. PCR-single-strand conformational polymorphism (PCR-SSCP) has been developed for use in the rapid detection of point mutations (Orita et al., 1989). This technique is based on the observation

that in non-denaturing polyacrylamide gels, the electrophoretic mobility of single-stranded nucleic acid depends on its sequence in addition to its size. PCR-SSCP could be used for the detection of point mutations not only in the URR but in other HPV genes. Point mutations in the HPV E7 gene have been shown to affect transformation, transactivation and phosphorylation by the E7 protein (Storey et al, 1990a) and may influence virus oncogenic potential in vivo. Similarly, mutations in the E6 gene may affect E6 protein binding affinity to the p53 gene product or E2 mutations - the negative control of transcription.

Finally, PCR assays were designed to determine the physical state of HPV DNA in cervical lesions. HPV DNA has been found to be integrated into the host genome in the majority of cervical carcinomas and carcinoma-derived cell lines. The stage in the CIN-cancer sequence at which this event occurs is unclear but may be of importance in predicting which lesions are likely to progress. The results of some studies have suggested that integration occurs most commonly towards the more severe end of the CIN spectrum (Pater et al., 1986; Cullen et al., 1991). However, most studies have used a combination of Southern blotting and 2-dimensional electrophoresis for determination of physical state of HPV DNA. Some concern has been expressed regarding the accuracy of these techniques when applied to DNA extracted from small cervical biopsies (Pater et al., 1986). It is possible that a single copy of integrated DNA would not be detected due to the low concentration of DNA in some samples.

Several investigators have now designed PCR based assays for the determination of HPV physical state which obviate problems of low

concentration of DNA in cervical biopsies associated with the Southern blot approach. Das et al (1992) have used a PCR based assay as a complement to Southern blotting and two dimensional electrophoresis. With this method, the generation of a PCR amplified product using primers sited in the HPV E2 gene indicates the presence of episomal HPV DNA whereas, failure of PCR amplification is indicative of the presence of integrated HPV DNA only. The value of this approach as an additional complementary test is questionable as it does not allow for detection of HPV genomes integrated within HPV genes other than E2, such as E1 or L1 and could not detect integrated HPV DNA in the presence of the episomal form. A similar approach has been adopted by van den Brule et al (personal communication) although four pairs of HPV primers were employed for the individual PCR amplification of segments of the E1, E2, L2 and L1 genes.

One possible approach to the determination of physical state would be to use an adaptation of the whole genome PCR method (Kinzler and Vogelstein, 1989). Until recently this was not feasible due to constraints on the length of DNA fragment amplifiable by PCR. The recent development of automated heating blocks which have shorter ramp times is likely to extend the applicability of this approach.

In this study, PCR integration assays were designed for use on clinical material. However unlike other PCR assays the problems associated with both the possible integration of HPV DNA in genes other than E2 and the presence of integrated HPV DNA in the presence of the episomal form were taken into account. One potential problem of this assay may arise where both episomal and integrated DNA are present in the same sample

and episomal DNA predominates over integrated DNA at a ratio of greater than 5:1 which may tend to direct preferential amplification of the more concentrated target sequence.

The assay has been applied in its entirety to cloned HPV plasmid DNA, used as a mimic of integrated HPV and is likely to require some refinement for its application to clinical material. This assay represents a considerable improvement on other methods for the determination of physical state of HPV DNA. Once optimised, it may be used to clarify the stage in the CIN-cancer sequence at which the vital integration event most commonly occurs.

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